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THE

BIOLOGICAL BULLETIN

PUBLISHED BY
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VOLUME 155

AUGUST TO DECEMBER, 1978

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $8.00. Subscription per volume (three issues), $22.00.

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

THE BIOLOGICAL BULLETIN (ISSN 0006-3185)

Second-class-postage paid at Woods Hole, Mass., and additional mailing offices.

LANCASTER PRESS, INC., LANCASTER, PA.

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Number 1

THE
BIOLOGICAL BULLETIN

Marine Biological Laboratory
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AUG 29 1978
Woods Hole, Mass.

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LANCASTER PRESS, Inc.
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THE BIOLOGICAL BULLETIN

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THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed after the Literature Cited. Because of the high cost of setting such material in type authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

4. ***Literature Cited.*** The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of THE BIOLOGICAL BULLETIN, and must be typed *double-spaced* on separate pages. Note that citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list

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Vol. 155, No. 1 August, 1978

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:-

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

III. ARTICLES OF AMENDMENT

(On file in the office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meetings of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

> "No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.

> "Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.
>
> "The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the Bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI
Secretary of the Commonwealth)

IV. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised February 13, 1976)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting, either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed, with or without cause, by the affirmative vote of two-thirds of the Trustees then in office. Any Member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into

four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least 2 years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as a Trustee.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall elect a Treasurer and Clerk to serve one year, and Board Trustees as described in Article VIII (B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees then in office shall constitute a quorum. A lesser number than a quorum may adjourn any meeting from time to time without further notice.

XII. Any action required or permitted to be taken at any meeting of the Trustees or of the Executive Committee may be taken without a meeting if all the Trustees or members of the Executive Committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. Such a consent shall be treated for all purposes as a vote at a meeting.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote an amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. The Corporation will indemnify every person who is or was a trustee, officer or employee of the Corporation or a person who provides services without compensation to an Employee Benefit Plan maintained by the Corporation, for any liability (including reasonable costs of defense and settlement) arising by reason of any act or omission affecting an Employee Benefit Plan maintained by the Corporation or affecting the participants or beneficiaries of such Plan, including without limitation any damages, civil penalty or excise tax imposed pursuant to the Employee Retirement Income Security Act of 1974; provided, (1) that the Act or omission shall have occurred in the course of the person's service as trustee or officer of the Corporation or within the scope of the employment of an employee of the Corporation or in connection with a service provided without compensation to an Employee Benefit Plan maintained by the Corporation, (2) that the Act or omission be in good faith as determined by the Corporation (whose determination made in good faith and not arbitrarily or capriciously shall be conclusive), and (3) that the Corporation's obligation hereunder shall be offset to the extent of any otherwise applicable insurance coverage, under a policy maintained by the Corporation or any other person, or other source of indemnification (August 13, 1976).

V. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

"It is the first step in sociological wisdom, to recognize that the major advances in civilization are processes which all but wreck the societies in which they occur:—like unto an arrow in the hand of a child. The art of free society consists first in the maintenance of the symbolic code; and secondly in fearlessness of revision, to secure that the code serves those purposes which satisfy an enlightened reason. Those societies which cannot combine reverence to their symbols with freedom of revision, must ultimately decay either from anarchy, or from the slow atrophy of a life stifled by useless shadows."

Alfred North Whitehead—*Symbolism*

As it enters the last decade of its first century, the Marine Biological Laboratory must continue to consolidate earlier advances while redefining its objectives to meet the conspicuous needs and opportunities of the time. In the year just past the Laboratory has been extraordinarily rich in people—investigators, instructors, and students—and ideas. Inevitably, change is accompanied by personal and social insecurity; and the search for my successor has engendered anxieties. Happily such fears as may have emerged have already all but disappeared following the announcement on March 24 that Paul R. Gross, currently Graduate Dean and Professor of Biology at the University of Rochester, would become President and Director on July 1, 1978.

The richness of new opportunity, and the Laboratory's unique capacity to seize it, has been abundantly evident throughout the year. Some of our year-round research programs have been recognized as being among the nation's finest, dispelling the fears expressed within the Corporation just a few years ago that they would fail to measure up to the quality of our renowned summer programs. Thus, in its second report, the Visiting Committee to the Ecosystems Center (E. O. Wilson, Chairman) wrote, in part, "The projects in ecosystem analysis now underway at the Center are original, well conceived, and significant, and we consider them in the aggregate to constitute one of the best such research programs in the world."

The program of the Laboratory of Biophysics of the National Institute of Neurological and Communicative Disorders and Stroke now embraces almost every aspect of modern marine neurobiology from biophysical studies of squid and lobster axons to pioneering explorations of the neural basis of behavior. A vital underpinning of the behavioral studies has been the successful development of techniques for culturing marine nudibranchs.

No less significant are the loosely-knit, informal associations at the Laboratory; witness the several related programs in cell motility and morphogenesis, embracing the laboratories of Ray Stephens, Kenneth Edds, Shinya Inoué and Lewis Tilney, reenforced during the summers not only by more of the world's leaders in the field, but also by an increasing number of promising young investigators.

The continuing emergence of our year-round programs, coupled with the availability of funds to enable young investigators to spend summers at the Laboratory, have resulted in increased competition for space among summer investigators. We have looked forward to a time when the competition would become significant; at this writing it appears that not all qualified applicants will be accommodated in 1978. It seems likely that the problem will be more acute in 1979. Henceforth, the Research Space Committee will be required to set its priorities carefully, especially at the lower end of its scale, for with six investigators on the waiting list at this writing, and with the number of participants in the Steps Toward Independence Program (among others) scheduled to grow in 1979, we should anticipate a shortage of as many as fifteen spaces in 1979.

I am especially pleased to report that the number of able women, both investigators and students, applying for fellowships continues to increase. Leaving aside the Grass Fellowships, which are determined by the Foundation itself, and fellowships in courses, determined by instructors-in-charge (and not known to me at this writing), seventeen of thirty-four major fellowships at the Laboratory in the summer of 1978 have been awarded to women.

Education

Just five years ago the winter teaching at the Laboratory was centered almost entirely in the Boston University Marine Program. In earlier reports I have attested to BUMP's remarkable growth—more evident in quality than in numbers; thus, in this account I will stress the emergence of the Laboratory's own programs. In 1977–1978 over two hundred students will have participated in five January short-term courses and five intensive short courses.

The five January, 1978, courses were outstanding. The intellectual fare was rich and varied, highlighted by our new course in Comparative Pathology of Marine Invertebrates. All five courses will be offered again in January, 1979.

Dr. Morton Maser, who took up his responsibilities as Coordinator of Continuing Education on July 1, 1977, has responded in a way that could have been only dimly anticipated, organizing five one- and two-week residential laboratory courses in the winter and spring of 1977–1978. The imagination of enterprise and the devotion that he brings to his office give a new dimension to life at the Laboratory.

Included in the 1977–1978 offerings were programs in Optical Microscopy and Photomicrography in the Biomedical Sciences, with Robert D. Allen as Instructor-in-

Chief; Electron Microscopy in the Biological Sciences, headed by Blair Bowers and Maser; Stereology, Morphometry and Optical Analysis of Electron Micrographs, headed by Lee D. Peachy; and Computer Reconstruction of Three-Dimensional Biological Structures from Serial Sections, with Eduardo Macagno in charge, joined by Cyrus Levinthal and Peter Sterling. In addition, Maser presented two one-week courses in Electron Microscopy for Technicians.

Maser is constantly on the alert for short courses that cannot be, or at least have not been, incorporated into traditional academic programs, because of considerations either of tradition or of time and scheduling. Their characteristics describe the essence of continuing education in its original and exciting sense and fundamentally are similar to the bases upon which the Laboratory's summer courses are built. But although such short courses could be held in practically any building anywhere, their value is enhanced significantly by the astonishing web of associations that thrive at MBL.

The recently completed short course on Optical Microscopy and Photomicrography in the Biomedical Sciences provided examples of how the short course format and the Laboratory tradition are mutually beneficial. Sixteen student-participants (most of whom are actively engaged in research) gathered for a week surrounded by $500,000 worth of modern optical microscopical equipment and a faculty and support staff of about a dozen to learn not only "nuts and bolts" microscopy but also the most modern and experimental aspects of this science. In spite of the fact that the Instructor-in-Chief, Robert Allen, was physically incapacitated for much of the program, the course was innovative and was satisfactory and rewarding to the entire range of participants. One of the students was Larry Lewis of Millersville State College in Pennsylvania, who will be a Steps Toward Independence Fellow in 1978. It happened that Keigi Fujiwara, one of the guest faculty, also will be a Fellow in the same program. To quote a recent letter from Lewis, "One very tangible result is that I will be collaborating with Keigi Fujiwara this summer."

Nor have the summer courses been immune to change. Our new offering in 1978, Neural Systems and Behavior, with a nationally prominent staff headed by Alan Gelperin, deserves special mention. It replaces the Experimental Invertebrate Zoology course. An invertebrate zoology course has been one of the Laboratory's traditional offerings. In recent years—for about a decade, in fact—ours was a zoology course in name only. It was successively a comparative physiology course and a physiological ecology course. Thus, we decided to face the issue squarely and to rename the course to define its content as accurately as possible. 1978 will see relatively few other changes. David Epel, who has rendered splendid service in recent summers as Co-Instructor-in-Charge of Embryology had to relinquish his responsibilities; he has been replaced by Joan Ruderman. We have also had to say good-bye, again reluctantly, to A. O. W. Stretton, who has stepped aside as Co-Director of the Neurobiology course, which Edward Kravitz will continue to direct.

Management

One of the secrets of the success of our educational programs in 1977–1978 has been Lucena Barth's devoted and wise stewardship of our new Admissions Office. There is much to be said for retiring when one has completed a good year; and Lucena has done just that. She has done a remarkable job of coordinating all of our educational efforts, including not only the summer and winter courses but also the Josiah Macy, Jr. and Steps Toward Independence fellowship programs, developing new procedures for their promotion and for screening candidates. This newly organized office will now become a part of Dr. Maser's responsibilities.

Support: four years in review

This report covers the progress of our funding program for the period beginning January 1, 1974, through March 31, 1978. During this period the Laboratory received over $4.3 million in funds from private sources, this figure representing the total in grants and pledges from foundations, corporations and individuals. All told there were sixty-seven awards from forty-five different foundations and corporations, which are listed below. These awards should provide a firm base on which to build future campaigns. Priorities in the years immediately ahead have been set: capital funding for the Marine Resources Center and the renovation of the Lillie Building, and, equally important, a drive to increase the Laboratory's endowment.

Atlantic Richfield Foundation
Charles Ulrick & Josephine Bay Foundation
Robert Sterling Clark Foundation
The Clowes Fund
Commonwealth Fund
Josephine B. Crane Foundation
Charles E. Culpepper Foundation
Fred Harris Daniels Foundation, Inc.
Arthur Vining Davis Foundations
Henry L. and Grace Doherty Charitable Foundation
Eastern Associated Foundation
Exxon Corporation
Max C. Fleischmann Foundation
Friendship Fund
General Electric Foundation
Gillette Charitable & Educational Foundation
Grace Foundation, Inc.
Grass Foundation
Charles Hayden Foundation
William Randolph Hearst Foundation
Lillia Babbitt Hyde Foundation
IBM Corporation
Edward Bangs Kelley & Elizabeth Kelley Foundation
Charles A. King Trust
Josiah Macy, Jr. Foundation
Andrew W. Mellon Foundation
Richard King Mellon Foundation
Merck Company Foundation
Merck, Sharpe & Dohme Research Laboratories
Mobil Foundation
NL Industries
Jessie Smith Noyes Foundation
Pew Memorial Trust
Pfizer, Inc.
Raytheon Charitable Foundation
Fannie E. Rippel Foundation
Rockefeller Foundation
Rowland Foundation, Inc.
Sandoz Foundation
William E. & Bertha E. Schrafft Charitable Trust
Alfred P. Sloan Foundation
Seth Sprague Educational & Charitable Foundation
Surdna Foundation
UPS Foundation
Edwin S. Weber Foundation

1. The Staff

ECOLOGY

I. Instructors

John M. Teal, Woods Hole Oceanographic Institution, co-director of course
Ivan Valiela, Boston University Marine Program, co-director of course
Kenneth H. Mann, Dalhousie University
William Odum, University of Virginia
Karen Porter, University of Michigan
James Porter, University of Michigan
Thomas Fenchel, University of Aarhus, Denmark
J. Rudi Strickler, Yale University
Charles Hall, Cornell University
Charlene Van Raalte, Hampshire College

II. Assistant

Mike Petrovski, Boston University Marine Program

III. Special Lecturers

Howard Sanders, Woods Hole Oceanographic Institution
Richard Haedrich, Woods Hole Oceanographic Institution
Charles Hollister, Woods Hole Oceanographic Institution
Peter Wiebe, Woods Hole Oceanographic Institution
Laureh Haury, Woods Hole Oceanographic Institution
Frank Carey, Woods Hole Oceanographic Institution
Larry Madin, Woods Hole Oceanographic Institution
George Grice, Woods Hole Oceanographic Institution
Joy Geiselman, Woods Hole Oceanographic Institution
Arthur Humes, Boston University Marine Program
Susan Vince, Boston University Marine Program
Bruce Peterson, The Ecosystems Center
John Hobbie, The Ecosystems Center
John J. McCarthy, Harvard University

IV. Lectures

J. Teal	Introduction to the oceans
J. Teal	Beaches and dunes
J. Teal	The ARGO MERCHANT oil spill
I. Valiela	Structure and function of salt marshes
I. Valiela	Nutrient budgets in a salt marsh
I. Valiela	Experimental studies of salt marsh pollution
R. Haedrich	The nature or oceanic fishes
R. Haedrich	Distribution and natural history of deep benthic fishes
B. Peterson	Primary production laboratory
R. Howarth	The role of sulfur in salt marsh metabolism
H. Sanders	Patterns of recovery in the aftermath of the West Falmouth oil spill
P. Wiebe	The biology of cold core rings
L. Haury	Zooplankton swimming: energetics and other consequences
T. Fenchel	Detritus food chain in aquatic environments
T. Fenchel	The biology of the sulfur cycle
J. Porter	Life in three dimensions: the structure of open ocean communities in contrast to terrestrial ecosystems
J. Porter	Life in two dimensions: the structure of communities in substrate-bounded marine ecosystems
J. Porter	Resource partitioning in space and time for marine ecosystems
K. Porter	Filter feeding as a way of life
K. Porter	Grazing and planktonic community structure
S. Vince	Plant-herbivore interactions in a salt marsh
J. R. Strickler	Zooplankton: life at random?
J. R. Strickler	Orientation of planktonic crustaceans
J. R. Strickler	Do behavior and morphology separate niches?
J. Hobbie	Structure of arctic tundra pond ecosystems
F. Carey	Ecology of tuna
G. Grice	The whale problem
G. Grice	The effects of mercury on pelagic ecosystems: giant bag experiments
L. Madin	The biology of gelatinous plankton

K. H. Mann	Studies on the productivity of kelp in Nova Scotia
K. H. Mann	Seaweeds, sea urchins and lobsters: a problem in resource management
D. Botkin	Stability and diversity in ecosystems
K. H. Mann	Some key roles for microbes in coastal ecosystems
J. J. McCarthy	The nitrogenous and phosphorus nutrition of ocean phytoplankton—or, getting something from nothing
W. Odum	The ecology of mangroves
W. Odum	Detritus feeding in coastal ecosystems
W. Odum	Management of coastal environments
J. Geiselman	Chemical defenses in marine macroalgae
C. Hall	Nutrient dynamics in estuaries
C. Hall	Energetics analysis of salmon ecosystems

EMBRYOLOGY

I. Instructors

Tom Humphreys, University of Hawaii, co-director of course
David Epel, University of California, San Diego, co-director of course
Stanley Cohen, Vanderbilt University
R. K. Hunt, The Johns Hopkins University
Tim Hunt, University of Cambridge, England, U.K.
Birgit Rose, University of Miami

II. Assistants

W. Steven Adair, Wesleyan University
Randal Gerber, University of Hawaii
Nancy Wright, Duke University
Marc Laufer

III. Lectures

D. Epel	Fertilization reactions
D. Epel	Regulation of metabolic activity at fertilization
T. Humphreys	Activation of protein synthesis at fertilization
T. Hunt	Globin synthesis in reticulocytes
T. Hunt	Regulation of the translation of globin mRNA
R. K. Hunt	Development of the *Xenopus* eye
R. K. Hunt	Development of nerve patterns
B. Rose	Cell communication *via* junctional membrane: regulation by cytoplasmic free Ca^{++}
T. Humphreys	Biochemical studies on aggregation of marine sponges
S. Cohen	Characteristics of growth factors
S. Cohen	Interaction of epidermal growth factor with cells
T. Humphreys	Ribosomal RNA synthesis and growth of sea urchin embryos
W. S. Adair	Cell surface changes associated with mating interactions of *Tetrahymena*
J. W. Saunders	Signaling systems in vertebrate limb development
V. Goodenough	Genetic control of gametic differentiation in *Chlamydomonas reinhardii*
J. P. Trinkaus	Surface interactions of gastrula cells during locomotion

W. Wasserman	Cytoplasmic control of meiotic maturation in amphibian oocytes
L. Jaffe	Electrical controls of development
D. Friend	Morphological studies of membrane interactions: fusions, junctions, and controls
R. Steinhart	Calcium and pH transients at activation in the sea urchin egg
J. Gerhart	Protein phosphorylation in the control of maturation of amphibian oocytes
H. Kanatani	Hormones controlling starfish oocyte maturation
A. Monroy	Sperm-egg interactions in ascidian fertilization
L. Jaffe	Fertilization of voltage-clamped sea urchin eggs
H. Kress	Regulation of puff formation in *Drosophila* salivary chromosomes
A. Warner	Changes in membrane properties during specification of the nervous system
A. Warner	Cell to cell communication in the early amphibian embryo

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. Instructors

Michael J. Greenberg, Florida State University, director of course
Stephen H. Bishop, Baylor College of Medicine
Richard J. Hoffmann, University of Pittsburgh
William R. Kem, University of Florida
Linda H. Mantel, The City University of New York
Sidney K. Pierce, Jr., University of Maryland
David J. Prior, University of Kentucky
J. Malcolm Shick, University of Maine

II. Consultants

F. A. Brown, Jr., Northwestern University
James Case, University of California, Santa Barbara
Robert K. Josephson, University of California, Irvine
Charlotte P. Mangum, College of William and Mary
C. Ladd Prosser, University of Illinois
W. D. Russell-Hunter, Syracuse University
Thomas J. M. Schopf, University of Chicago

III. Assistants

Christina Myles, Manchester Community College
Raymond Fetterer, Jr., Michigan State University

IV. Secretary

Tonicia M. Smith, Marine Biological Laboratory

V. Special Lecturers

John Arnold, University of Hawaii
Peter Calow, University of Glasgow, Scotland, U.K,

Edward J. Carroll, Jr., University of California, Riverside
A. Farmanfarmaian, Rutgers—The State University of New Jersey
G. Richard Harbison, Woods Hole Oceanographic Institution
Dennis J. Murphy, Connecticut College
C. Ladd Prosser, University of Illinois

VI. Lectures

M. J. Greenberg	Introductory notes
R. J. Hoffmann	Population and genetic structure of marine invertebrates
R. J. Hoffmann	Environment and biochemical polymorphism
R. J. Hoffmann	Cape Cod environment
R. J. Hoffmann	Physiological possibilities of multiple molecular forms
G. R. Harbison	Jello plankton: life in the open ocean
S. K. Pierce, Jr.	Salinity, osmosis, diffusion and, briefly, membrane structure
S. K. Pierce, Jr.	Mechanisms of salinity tolerance: cellular volume regulation
S. K. Pierce, Jr.	Water balance: functional morphology of excretory systems
L. H. Mantel	Ionic regulation: the problem and its solutions
L. H. Mantel	Cellular mechanisms and hormonal control of ionic regulation
A. Farmanfarmaian	Membrane transport of sugars and amino acids in intestinal epithelial—some hard facts and popular speculations
M. J. Greenberg	The distribution of animals with salinity: the horohalinicum as a physiological island
M. J. Greenberg	Circulation: compartmentalization and filtration
M. J. Greenberg	Circulation: volume, pressure and flow
S. H. Bishop	Control of amino acid pool sizes
S. H. Bishop	Physiological role of ammonia
J. M. Shick	Ecological physiology and genetics of the colonizing sea anemone, *Haliplanella luciae*
S. H. Bishop	Nitrogen detoxification and phosphagens
S. H. Bishop	Metabolic adaptations and facultative anaerobiosis
S. K. Pierce, Jr.	Water balance: a look at some intact animals
J. M. Shick	Principles of gas exchange; body size, the limitations of diffusion, and respiratory surfaces
J. M. Shick	Respiratory responses to declining P_{O_2}; external and internal convection
J. M. Shick	Ecological and bioenergetic aspects of oxygen supply and consumption
E. J. Carroll, Jr.	Cell surface events during sea urchin fertilization
J. M. Shick	Invertebrate respiratory pigments
J. Arnold	Squid mating demonstration
W. R. Kem	Nemertine toxins
W. R. Kem	Coelenterate toxins
W. R. Kem	Annelid, molluscan, and echinoderm toxins
W. R. Kem	Invertebrate digestion: proteases and protease inhibitors
S. H. Bishop	Phosphoglycoprotein connective tissue components in coelenterates and other invertebrates

D. J. Murphy	The physiological basis for change in the freezing tolerance of a bivalve mollusc
D. J. Murphy	Metabolic adaptations of ectothermic animals to temperature changes
L. H. Mantel	Introduction to neuroendocrine systems
L. H. Mantel	Neuroendocrine mechanisms in the daily life of invertebrates
L. H. Mantel	Neuroendocrine regulation of cyclic phenomena in invertebrates
P. Calow	Gaining time by losing weight; or can a flatworm live forever?
D. J. Prior	Nervous systems, neurons and effectors
D. J. Prior	Integration: mechanisms of interaction between neurons
C. L. Prosser	Role of the central nervous system in temperature adaptation
D. J. Prior	Neuroethology, I and II
D. J. Prior	Neural basis of adaptive behavioral modifications

EXPERIMENTAL MARINE BOTANY

(Comparative Biology and Biochemistry of Algae)

I. Instructors

Jerome A. Schiff, Brandeis University, director of course
James Fiore, Suffolk University, laboratory instructor
David Mauzerall, The Rockefeller University
Robert D. Simon, University of Rochester, laboratory instructor
Robert Troxler, Boston University

II. Consultants

Robert L. Guillard, Woods Hole Oceanographic Institution
Frank A. Loewus, Washington State University
Ralph S. Quatrano, Oregon State University

III. Staff Associates

Hans Gaffron, Woods Hole and Sanibel, Florida
Aimlee Laderman, State University of New York at Binghamton

IV. Assistants

Carol Kenny, University of Rochester
Michael Mishkind, State University of New York at Stony Brook
Scott Schatz, University of Rhode Island

V. Special Lecturers

Franklin Fong, Brandeis University
Martin Gibbs, Brandeis University
Jane Gibson, Cornell University
Zipporah Gromet-Elchanon, The Weizmann Institute
Alfred Loeblich, Harvard University
Harvard Lyman, State University of New York at Stony Brook

JOSEPH RAMUS, Yale University
JAMES SEARS, Southern Massachusetts University
RAYMOND E. STEPHENS, Marine Biological Laboratory
ARTHUR STERN, University of Massachusetts, Amherst
ROGER THORNELEY, Sussex University, U.K.

VI. LECTURES

J. A. SCHIFF	Chemical phase of evolution biogeochemistry
J. A. SCHIFF	The appearance of oxygen
J. A. SCHIFF	Evolution of procaryotes
J. A. SCHIFF	Evolution of eucaryotes and organelles
J. A. SCHIFF	Evolution of life cycles
J. A. SCHIFF	Nutritional cycles
J. A. SCHIFF	Metabolism of nitrogen and sulfur
R. TROXLER	Biosynthesis of heme and chlorophyll, early stages
R. TROXLER	Biosynthesis of heme and chlorophyll, later stages
R. TROXLER	Open chain tetrapyrroles
J. GIBSON	Photosynthetic electron transport
A. STERN	Photophosphorylation
Z. GROMET-ELCHANON	Coupling factor ATPase complex of photosynthetic bacteria and its relation to proton transport
D. MAUZERALL	Photochemical principles
D. MAUZERALL	Photochemistry of photosynthesis
J. SEARS	Vertical distribution of subtidal algae
J. RAMUS	Chromatic adaptation in benthic algae
J. FIORE	Life histories of brown algae
R. THORNELEY	Nitrogenase
R. SIMON	Plasmids and nucleic acid
R. SIMON	Cyanophycin granules
J. A. SCHIFF	Structure and continuity of chloroplasts and mitochondria
J. A. SCHIFF	Development of chloroplasts and mitochondria
J. A. SCHIFF	Photoregulation of plastid development
M. GIBBS	Photoproduction of hydrogen
A. LOEBLICH	Unique cytology and molecular biology of dinoflagellates
F. FONG	Phototaxis in *Euglena*
R. E. STEPHENS	Microtubules in plant development
H. LYMAN	Symbiosis of algae and chloroplasts with animal cells
R. SIMON	Culture techniques
J. FIORE	Field and preservation methods
J. FIORE	Chlorophyta, I, II and III
R. SIMON	Pigment methodology
R. SIMON	Photosynthesis methodology
J. A. SCHIFF	Development of single cells from *Prasiola*
J. A. SCHIFF	Euglenophyta
R. SIMON	Cyanobacteria (Blue Green Algae), I and II
R. SIMON	Nitrogen fixation methodology
R. SIMON	Cyanophages
R. SIMON	Phycobilin methodology
J. FIORE	Pheophyta, I, II and III
J. FIORE	Rhodophyta, I, II and III
J. FIORE AND R. SIMON	Chrysophyta, Xanthophyta
J. FIORE AND R. SIMON	Bacillariophyta

J. Fiore and R. Simon	Pyrrophyta
J. Fiore	Algal ecology, I
R. Guillard	Algal ecology, II and III

NEUROBIOLOGY

I. Instructors

Edward A. Kravitz, Harvard Medical School, co-director of course
Antony O. W. Stretton, Wisconsin University, co-director of course
Barbara A. Battelle, Harvard Medical School
Jonathan B. Cohen, Harvard Medical School
Gerald D. Fischbach, Harvard Medical School
Edwin J. Furshpan, Harvard Medical School
John Heuser, University of California, San Francisco
Paul H. O'Lague, University of California, Los Angeles
David D. Potter, Harvard Medical School
Thomas S. Reese, National Institutes of Health
Michel Weber, Harvard Medical School

II. Assistants

Sue Huttner, University of California, Los Angeles
Margaret Livingstone, Harvard Medical School
Mary Rheuban, Pennsylvania State University
Ruth Siegel, Harvard Medical School

III. Special Lecturers

Daniel Branton, Harvard University
Robert Campenot, Harvard Medical School
Don Fischman, State University of New York, Downstate Medical Center
Luis Glaser, Washington University
Stan Goldin, Harvard Medical School
Guido Guidotti, Harvard University
Juan I. Korenbrot, University of California, San Francisco
Mahlon Kriebel, State University of New York, Upstate Medical Center
Raymond J. Lasek, Case Western Reserve University
Cyrus Levinthal, Columbia University
Rodolpho Llinas, New York University
Harvey Pollard, National Institutes of Health
Schlomo Rotshenker, Harvard University
Hennig Stieve, Institut fur Neurobiologie der KFA Julich
Walter Stoeckenius, University of California, San Francisco
Kunitaro Takahashi, Tokyo University

IV. Lectures

T. S. Reese	General cytological features of nervous systems
T. S. Reese	Nonsynaptic junctions in the nervous system
J. E. Heuser	Structure of synapses: presynaptic processes, I and II
T. S. Reese	Structure of synapses: the postsynaptic membrane
J. E. Heuser	Chromatolysis and axoplasmic flow
T. S. Reese	Formation of synapses: cytological aspects

G. D. Fischbach	Aspects of nerve and muscle cell differentiation (electrical excitability)
G. D. Fischbach	Development of chemoreceptors (ACh)
J. B. Cohen	Isolation of functional postsynaptic membrane in electric fish
G. D. Fischbach	Synapse formation: early events
G. D. Fischbach	Synapse formation: selectivity
J. B. Cohen	Biochemical characterization of cholinergic receptors
G. D. Fischbach	Stability of synapses in development
M. Weber	Development of neural crest
A. O. W. Stretton	Acetylcholine
E. A. Kravitz	GABA
A. O. W. Stretton	Catecholamines
E. A. Kravitz	CNS, I. Amines and synaptic modulation
B. A. Battelle	Cyclic nucleotides
E. A. Kravitz	CNS, II. Peptides
G. Guidotti	Structure of membrane transport proteins
D. Branton	Control of macromolecular mobility in the erythrocyte membrane
L. Glaser	Membranes as a tool to study cell-cell interactions
W. Stoeckenius	Function of purple membrane in *Halobacterium halobrium*
J. I. Korenbrot	Reconstitution of membrane transport functions in planar interface films
S. Goldin	Resolution *in vitro* of the molecular identity of the Na-K ATPase driven active transport system
K. Takahashi	Intracellular perfusion of the tunicate egg and the effect of ionized calcium upon both Na^+ and Ca^{++} channels
J. Brown	Intracellular calcium in *Limulus* photoreceptors
M. Bennett	Electrical transmission
H. Stieve	The dependence of the electrical light response of *Limulus* photoreceptors on extracellular calcium concentrations
R. Llinas	Calcium and transmitter release at the squid stellate ganglion
M. Kriebel	Quantal release at the neuromuscular junction: nature of the small mode and Schwann cell MEPPs
C. Levinthal	Shape and development of identified cells in identical twins
R. J. Lasek	How do axons stop growing? Some answers from the slow component of axonal transport
R. Campenot	The local control of neurite growth by NGF
S. Rotshenker	Specific reinnervation of old endplates
D. Fischman	Immunological studies of embryonic muscle membranes
H. Pollard	Anion hypothesis for regulation of exocytosis
A. O. W. Stretton	Anatomy, physiology, and chemistry of the motoneurons of *Ascaris*
R. Rahamimoff	Calcium and transmitter release
T. Wiesel	The processing of visual information
P. Patterson	Sympathetic neurons in culture

PHYSIOLOGY

I. Instructors

K. E. Van Holde, Oregon State University, director of course
Thomas Baldwin, University of Illinois

Jay Brown, University of Virginia
Leonard Cohen, Institute for Cancer Research
Benjamin Hall, University of Washington
Lynn Klotz, Harvard University
Raymond Stephens, Brandeis University

II. Consultants

Kimball C. Atwood III, Columbia University
Irvin Isenberg, Oregon State University

III. Staff Associates

James Anderson, Harvard University
Jeff Bennetzen, University of Washington
Al Christopher, University of Virginia
Laurel Loeblich, Harvard University
Mark Mooseker, Harvard Medical School
Kenneth Newrock, Institute for Cancer Research
Miriam Nicoli, University of Illinois
Kelley Tatchell, Oregon State University

IV. Assistants

Martha Barrett, University of Colorado at Denver
Patric Galvin, University of Colorado at Denver
David Von Hipple, University of Oregon

V. Special Lecturers

Kimball C. Atwood III, Columbia University
John Cameron, Stanford University
Alfred Goldberg, Harvard University
Lowell Hager, University of Illinois
H. O. Halvorson, Brandeis University
J. W. Hastings, Harvard University
Teru Hayashi, Illinois Institute of Technology
W. C. Johnson, Oregon State University
Alex Keynan, Hebrew University, Israel
David Lilley, Searle Research Laboratory
Lazlo Lorand, Northwestern University
Kenneth Nealson, Scripps Institution of Oceanography
Thomas Petes, Harvard University
Thomas Pollard, Harvard University
Jeff Rosen, Baylor College of Medicine
Joel Rosenbaum, Yale University
Ruth Sager, Harvard Medical School
John Scocca, The Johns Hopkins University
Paul Sigler, University of Chicago
James Spudich, University of California, San Francisco
John Spudich, Harvard Medical School
Andrew Szent-Gyorgyi, Brandeis University
O. Uhlenbeck, University of Illinois
Eric Weinberg, The Johns Hopkins University
J. Wooley, Harvard University

VI. Lectures

K. E. Van Holde	Chromatin structure, I and II
D. Lilley	Structure of chromatin cores
B. Hall	Transfer RNA genes in yeast
I. Isenberg	Nucleoprotein interactions, I and II
J. Wooley	STEM electron microscopy and neutron diffraction of chromatin
T. Baldwin	Techniques of peptide mapping and purification
T. Baldwin	Techniques of amino acid analysis and sequence determination
L. Cohen	Chromatin remodeling in development, I and II
J. Spudich	Actin and myosin, molecules in motion
J. Spudich	Bacterial hemotaxis and nongenetic individuality
L. Lorand	Molecular aspects of blood coagulation
T. Petes	Genetic analysis of yeast ribosomal DNA
T. Pollard	Contractile mechanisms in muscle and nonmuscle cells
L. Hager	Studies on halogenated compounds in marine organisms
A. Szent-Gyorgyi	Regulation of muscle contraction
R. Stephens	Microtubules, I and II
J. Rosenbaum	Control of flagellar growth
M. Mooseker	The association of actin with membranes
J. Scocca	Role of the bacterial cell surface in genetic transformation
T. Hayashi	Actin-myosin interaction
P. Sigler	Studies on the structure of initiator t-RNA-synthetase complex
O. Uhlenbeck	Quantitative *in situ* hybridization
J. Brown	Animal cell membranes: the protein components
J. Brown	Animal cell membranes: the phospholipids
L. Klotz	DNA organization in two primitive eukaryotes: dinoflagellates and yeast, I and II
L. Klotz	A unified theory of DNA renaturation
K. C. Atwood	Limitation of genetic diversity in cell population, I and II
F. Goldberg	Control of protein degradation
K. Nealson	Physiology of bioluminescent bacteria
T. Schuster	Kinetics of protein subunit interactions
J. W. Hastings	Bioluminescence and chemiluminescence
T. Baldwin	Bacterial luciferase
W. C. Johnson	Circular dichroism
A. Keynan	Cell cycle dependence of induction of sporulation in *B. subtilis*
H. O. Halvorson	Amplification of ribosomal RNA genes in yeast
R. Sager	Genetic studies with mammalian cells
B. Hall	Structure and function of yeast t-RNA genes
J. Cameron	Isolation of yeast t-RNA genes by molecular cloning: structural analysis by electron microscope
E. Weinberg	Sea urchin histone genes
J. Rosen	Use of c-RNA probes for quantitation of m-RNA genes
L. Cohen	Optical monitoring of neuron activity
E. Szuts	Vertebrate photoreceptors
A. Fein	Role of intracellular sodium in light adaptation
F. Lang	Synaptic transmission—the soups *vs.* the sparks
F. Lang	Transmitter release; ionic basis of synaptic potentials

F. Lang	Quantal release
F. Lang	Excitation-secretion coupling
R. Hoy	Cricket song: analysis of an animal communication system
F. Lang	Origin and fate of synaptic vesicles
F. Lang	Electrical synapses
U. J. McMahan	Topographic specificity at the regenerating neuromuscular junction
E. Frank	Specificity of peripheral synapse formation
E. Frank	Formation and development of nerve-muscle synapses in tissue culture
F. Lang	Physiological basis for changes in fight and flight behavior during growth of the lobster
E. A. Kravitz	Three neurohormones in the lobster: studies on the cellular localization, release and physiological actions of octopamine, serotonin, and dopamine
C. M. Lent	Organizational and neural properties of leech CNS
A. Fein	Crustacean stretch receptor
Y. Grossman	Conduction of action potentials along nonhomogenous axons
D. L. Alkon	Associative training in a nudibranch mollusc, *Hermissenda*
E. Szuts	Vertebrate proprioreception
R. J. French	Axon channels: asymmetry and selectivity

YEAR-ROUND PROGRAMS
1977

BOSTON UNIVERSITY MARINE PROGRAM

The Staff

Arthur Humes, Boston University Marine Program, director
Jelle Atema, Boston University Marine Program
Björn Ganning, University of Stockholm, Sweden (Semester I only)
Stjepko Golubic, Boston University (Semester II only)
Frederick Lang, Boston University Marine Program
Charles Lent, State University of New York at Stony Brook (Semester II only)
Ivan Valiela, Boston University

THE ECOSYSTEMS CENTER

George M. Woodwell, director

John E. Hobbie, senior scientist; Daniel B. Botkin, associate scientist; Jerry M. Melillo, Bruce J. Peterson, assistant scientists; Cameron E. Gifford, field research coordinator; Richard H. Burroughs, senior fellow; John T. Finn, postdoctoral fellow; Erene V. Pecan, assistant to the director and research associate; Richard A. Houghton, Paul A. Steudler, research associates; John V. Helfrich, David S. Schimel, research assistants; James T. Morris, William B. Bowden, James P. Reed, graduate research assistants; Maybelline L. Philbin, taxonomist; Jeffery B. Shelkey, field assistant; Joan M. Upton, secretary; Frances R. Seymore, typist

JANUARY COURSES 1977

BEHAVIOR

(Offered Jointly by Boston University Marine Program and the Marine Biological Laboratory)

I. Instructors

Jelle Atema, Boston University, director of course
Adrianus J. Kalmijn, Woods Hole Oceanographic Institution
Stewart Jacobson, Boston University
Daniel Alkon, NIH-NINCDS, Woods Hole
James Loy, University of Rhode Island
Douglas Smith, State University of New York at Stony Brook
Ivan Valiela, Boston University
Beverly Greenspan, Bowdoin College
Stanley Fox, Boston University
Margaret Nelson, Harvard Medical School
John Palmer, University of Massachusetts
Bori Olla, Sandy Hook Laboratory
Katy Payne, The Rockfeller University and N. Y. Zoological Society
Roger Payne, The Rockefeller University and N. Y. Zoological Society
Noel Snyder, Patuxent Wildlife Research Center
Timothy Williams, Swarthmore College
George Michel, Boston University
I. Lehr Brisbin, Savannah River Ecology Program
Ronald Larkin, The Rockefeller University
Carl Berg, Marine Biological Laboratory
Dietland Müller-Schwartze, State University of New York at Syracuse

II. Assistants

Daniel Stenzler, Boston University
Vera Kalmijn, Woods Hole Oceanographic Institution

III. Lectures

J. Atema	Introduction
J. Atema	Sensory physiology and behavior
A. J. Kalmijn	Electroreception with and without electric organs
J. Atema	Evolution of chemical senses
A. J. Kalmijn	Electroreception: the detection of inanimate electric fields
J. Atema	Chemo- and mechanoreceptors
A. J. Kalmijn	The physics and physiology of electroreception
I. Valiela	Principles of experimental design in ecology and behavior
A. J. Kalmijn	Electroreception: more questions with or without answers
K. Payne	Annual changes in songs of humpback whales
R. Payne	The possibility of long-range communication between Baleen whales
R. Payne	The southern Right whale
N. Snyder	Limpkins, everglade kites, and apple snails: adaptation for and against predation
N. Snyder	Preserving an almost extinct species: the Puerto Rican parrot

G. Michel	Introduction to ethology
G. Michel	Role of learning in behavior
G. Michel	Hormones and behavior
B. Olla	Natural history and behavior of marine fishes: the importance of defining habits under natural conditions
B. Olla	Comparative aspects of the responses of marine fishes to environmental stress
B. Olla and J. Atema	Tunas and pelagic fish: schooling behavior
I. L. Brisbin	Ecology, domestication and the behavior of dogs: some principles and their application to present-day problem of men, wolves and dogs
I. L. Brisbin	The domestic dog today: training and tracking
J. Palmer	Introduction to biological rhythms
J. Palmer	Biological rhythms in shore dwelling animals
J. Palmer	Human rhythms
R. Larkin	Introduction to bird orientation and navigation
R. Hoy	Genetics, physiology and behavior of singing crickets
T. Williams	Some thoughts on the evolution of bird migration
M. Nelson	Introduction to neural control of behavior
M. Nelson	Hissing and social behavior in cockroaches
B. Greenspan	Display and ritualization in courtship
B. Greenspan	Leks and other mating systems, particularly in arthropods
B. Greenspan	Male reproductive success in the communal courtship system of the fiddler crab, *Uca rapax*
J. Atema and S. Jacobson	Social behavior of the lobster, *Homarus americanus*
S. Fox	Introduction to territoriality, aggression and dispersal
S. Fox	Natural selection among behavioral phenotypes in lizard
D. Smith	Introduction to social behavior in birds
D. Smith	Acoustic and visual signals in redwing blackbirds
D. Müller-Schwarze	Predator-prey relations in Antarctic bird communities
D. Müller-Schwarze	Introduction to pheromones
D. Müller-Schwarze	Mammalian pheromones
J. D. Ebert	Ionic regulation of embryonic development
J. Loy	Introduction to primate sociobiology
J. Loy	Old world monkeys I: Patas and the one-male group
J. Loy	Old world monkeys II: Rhesus and the multi-male group
A. Stuart	Social behavior of termites
A. Stuart	Termite communication
S. MacKay	Bio-medical telemetry in behavioral studies
D. Alkon	Associative training in a nudibranch mollusc, *Hermissenda*
D. Alkon	Neural substrates of associative training in *Hermissenda*
J. Todd	The new alchemy

DEVELOPMENTAL BIOLOGY

I. Instructors

Walter S. Vincent, University of Delaware, director of course
James D. Ebert, Marine Biological Laboratory
Kenneth Edds, Marine Biological Laboratory
Shinya Inoué, University of Pennsylvania
William H. Massover, Brown University
Raymond E. Stephens, Brandeis University

II. ASSISTANT

CHARLES HENRY, Washington and Jefferson College

III. SPECIAL LECTURERS

EVERETT ANDERSON, Harvard University
JACK COLLIER, Brooklyn College, The City University of New York
LOUIS DELANNEY, Ithaca College
DAVID FRANCIS, University of Delaware
SUSAN GERBI, Brown University
HARLYN HALVORSON, Brandeis University
JUNE HARRIGAN, Marine Biological Laboratory
ARTHUR HUMES, Boston University Marine Program
HANS LAUFFER, University of Connecticut
KEITH PORTER, University of Colorado and Marine Biological Laboratory
RICHARD TASCA, University of Delaware

IV. LECTURES

J. D. EBERT	Introduction to the Marine Biological Laboratory
W. S. VINCENT	Introduction to course
W. S. VINCENT	Origin of germ cells, gonad differentiation
W. S. VINCENT	Spermatogenesis
W. H. MASSOVER	Oogenesis, I and II
W. H. MASSOVER	Vitellogenesis
W. H. MASSOVER	Maturation of the oocyte
L. E. DELANNEY	Amphibian development
L. E. DELANNEY	Unsolved "classical problems" in development
L. E. DELANNEY	Immunological recognition and response
L. E. DELANNEY	Histo-incompatability among invertebrates
R. PAYNE	Whales
W. S. VINCENT	Egg membranes
W. S. VINCENT	Fertilization
S. INOUE	Mitosis, I and II
S. INOUE	Theories of chromosome movement
R. E. STEPHENS	Chemistry of microtubles
S. INOUE	Spicule formation and structure
R. E. STEPHENS	Ciliogenesis
R. E. STEPHENS	Cilia, synthesis or assembly?
K. T. EDDS	Patterns of cleavage
K. T. EDDS	Mechanics of cleavage, I and II
K. T. EDDS	Cellular morphogenesis in sea urchins
P. B. REDFIELD	Development of the zebra fish
R. HOY	Cricket song, analysis of an animal communication system
S. GERBI	Molecular cytogenetics and gene regulation, I and II
J. R. COLLIER	Gene expression in *Illyanassa* development, I and II
A. G. HUMES	Larval development in the Copepoda
H. LAUFFER	Gene expression in insect morphogenesis
W. S. VINCENT	Conservation of gene sequence in phylogeny
R. TASCA	Early mammalian morphogenesis
R. TASCA	Early biochemical events in mammalian development
H. O. HALVORSON	Sporulation as a model for development
D. FRANCIS	Genetics of development in cellular slime molds

J. D. Ebert	Birth defects and interacting systems in development
P. M. Fye	Law of the sea
J. D. Ebert	Membrane permeability and ion movement in development
R. E. Stephens	Membranes and morphogenesis
K. R. Porter	Cytoarchitecture
E. Anderson	Development of the graafian follicle

ECOLOGY

I. Instructors

George M. Woodwell, The Ecosystems Center, director of course
Daniel B. Botkin, The Ecosystems Center
Charles A. S. Hall, The Ecosystems Center
Jerry M. Melillo, The Ecosystems Center
Bruce J. Peterson, The Ecosystems Center

II. Assistants

William B. Bowden, The Ecosystems Center
Susan D. Cooley, The Ecosystems Center
John V. K. Helfrich, The Ecosystems Center
James P. Reed, The Ecosystems Center

III. Special Lecturers

Kenneth O. Emery, Woods Hole Oceanographic Institution
Paul M. Fye, Woods Hole Oceanographic Institution
Richard C. Hennemuth, National Marine Fisheries Service
Holger W. Jannasch, Woods Hole Oceanographic Institution
Marilyn J. Jordan, The Ecosystems Center
Thomas E. Lovejoy, World Wildlife Fund
Roger S. Payne, New York Zoological Society
Howard L. Sanders, Woods Hole Oceanographic Institution
Lawrence B. Slobodkin, State University of New York at Stony Brook
Frederick E. Smith, Harvard University
John M. Teal, Woods Hole Oceanographic Institution
John Todd, New Alchemy Institute
Ivan Valiela, Boston University
Robert H. Whittaker, Cornell University
Garrison Wilkes, University of Massachusetts

IV. Lectures

G. M. Woodwell	The structure of the biosphere, I and II
L. B. Slobodkin	Evolution
L. B. Slobodkin	Group selection
H. L. Sanders	Natural communities and evolutionary strategies, I and II
K. O. Emery	Continental drift
G. M. Woodwell	Climates of the world
J. M. Teal	Oceanography, I and II
R. S. Payne	Whales
G. M. Woodwell	Primary production and the metabolism of the earth
G. M. Woodwell	The world carbon budget: the predominance of forests
J. E. Hobbie	The world carbon budget: the predominance of waters

J. E. Hobbie	Primary productivity in aquatic systems, I and II
C. A. S. Hall	Secondary productivity: I. New Hope Creek
C. A. S. Hall	Secondary productivity: II. Pacific salmon
C. A. S. Hall	Secondary productivity: III. Hudson River fisheries
D. B. Botkin	Succession and primary production
D. B. Botkin	Terrestrial secondary productivity
R. H. Whittaker	Gradient analysis
R. H. Whittaker	Community patterns
R. H. Whittaker	The evolution of communities
T. E. Lovejoy	The tropical forests
H. W. Jannasch	Deep sea microbiology, I and II
G. Wilkes	Saving genes for agriculture
C. A. S. Hall	Nutrient cycles
B. J. Peterson	Nutrient limitation in freshwater systems
G. M. Woodwell	Nutrient cycles and communities
M. J. Jordan	Industrial pollution
J. M. Melillo	Nutrient cycling in terrestrial systems: Hubbard Brook
J. M. Melillo	Nutrient cycles: nitrogen
I. Valiela	Nutrient cycling in a salt marsh
G. M. Woodwell	Ecosystems, energy and world politics
D. B. Botkin	The stability of ecosystems
D. B. Botkin	Endangered species
P. M. Fye	Law of the sea
K. O. Emery	Mineral resources of the deep sea and international politics
R. C. Hennemuth	The future of fish, I and II
F. E. Smith	Some aspects of human ecology
F. E. Smith	Urban ecology, I and II
J. Todd	The new alchemy
G. M. Woodwell	Biotic impoverishment and the threshold dilemma

NEUROBIOLOGY

(*Offered jointly by Boston University Marine Program and the Marine Biological Laboratory*)

I. Instructors

Alan Fein, Marine Biological Laboratory, director of course
Frederick Lang, Boston University Marine Program, director of course
Edward F. MacNichol, Jr., Marine Biological Laboratory, laboratory director
Frederick A. Dodge, The Rockefeller University
Ferenc Harosi, Marine Biological Laboratory
Charles H. Lent, State University of New York at Stony Brook

II. Assistant

Russell H. Hill, Boston University Marine Program

III. Special Lecturers

William J. Adelman, NIH-NINCDS, Woods Hole
Daniel L. Alkon, NIH-NINCDS, Woods Hole
Jelle Atema, Boston University Marine Program

Melvin J. Cohen, Yale University
Eric Frank, Harvard Medical School
Robert J. French, NIH-NINCDS, Woods Hole
Alfredo Gorio, The Rockefeller University
Yoram Grossman, NIH-NINCDS, Woods Hole
Ronald Hoy, Cornell University
Adrianus Kalmijn, Woods Hole Oceanographic Institution
Edward A. Kravitz, Harvard Medical School
Alexander Mauro, The Rockefeller University
U. J. McMahan, Harvard Medical School
Ete Szuts, Marine Biological Laboratory

IV. Lectures

F. Lang, A. Fein, E. F. MacNichol, Jr.	Introduction
A. Fein	Cell permeability and the plasma membrane
U. J. McMahan	Microscopic anatomy of the nervous system
U. J. McMahan	The structure of synapses
J. Atema	Evolution of chemical senses
M. J. Cohen	Evolution of neuron structure and function
A. Kalmijn	Field studies of navigation in magnetic fields
A. Fein	The resting membrane potential
A. Fein	Cable properties of neurons
A. Fein	Action potentials
A. Fein	Saltatory conduction in myelinated axons
W. J. Adelman	Neuronal current, channels, spaces and clefts
F. A. Dodge	Biophysics and behavior of *Limulus* vision, I and II

LABORATORY OF BIOPHYSICS, NINCDS–NIH

William J. Adelman, Jr., chief of the laboratory and head, section on neural membranes
Daniel L. Alkon, head, section on neural systems
Kenneth S. Cole, senior scientist; Jay Wells, staff scientist; David E. Goldman, guest worker; Robert J. French, visiting associate; Terry Crow, staff fellow; Izja Lederhendler, visiting fellow; Jonathan Shoukimas, Alan Hancock fellow; Ferenc I. Harosi, Thomas Jerussi, IPA fellows; Wesley Corson; Alan Kuzirian, Daniel Stenzler, guest workers

LABORATORY OF SENSORY PHYSIOLOGY

Edward F. MacNichol, Jr., director
Alan Fein, assistant scientist and deputy director; Barbara Ann Collins, senior laboratory technician; J. Sherwood Charlton, Ete Z. Szuts, NIH postdoctoral fellows; Ferenc I. Harosi, Assistant Professor of Physiology, Boston University; Harold E. Johnson, computer technician and programmer; Ishik C. Tuna, computer programmer; Joseph Levine, graduate student, Harvard University; Kathleen French, graduate student, Boston University Marine Program; Jane Vincent, Robert Enos, research assistants

NATIONAL FOUNDATION FOR CANCER RESEARCH

Albert Szent-Gyorgyi, director
Jane A. McLaughlin; Peter Gascoyne; Richard Meany; Ronald Pethig, lecturer University College of North Wales; John T. Lewis, Professor, University College

of North Wales; Stephen Bone, graduate student, University College of North Wales; Diana Clark, Katy Clark, Charity Smith, part-time glassware cleaners; Blenda Antonellis, Michael DiSpezio, temporary

INDEPENDENT YEAR-ROUND PROGRAMS

Raymond Stephens, principal investigator
Melanie Pratt; Edward D. Salmon

Shinya Inoué, principal investigator
Daniel P. Kiehart

Audrey E. V. Haschemeyer, principal investigator
Rita W. Mathews; Jennifer B. K. Nielsen; Michael A. K. Smith

Kenneth Edds, principal investigator
Patricia Wadsworth

Eric Ball, principal investigator

Behrus Jahan-Parwar, principal investigator
Susan Perritt

James Oschman, principle investigator
Betty Wall, principal investigator

Timothy Williams, principal investigator
Janet Williams; Bruce Cohen; Kathy Caldwell

Judith Grassle, principal investigator
Fern Birtwistle; Cynthia Lanyon-Duncan; Robin Gross

Ruth D. Turner, principal investigator
Carl J. Berg; Tim Cole; Scott Gallager; Brad Calloway, graduate student

Robert Rice, principal investigator
Priscilla Roslansky; Susan Case; Ann Cornell-Bell; Judith Heldman; Natalie Pascoe

YEAR-IN-SCIENCE
1976–1977

The Year-in-Science program at the MBL is for advanced undergraduates and beginning graduate students. For undergraduates the program is equivalent to a university honors program; for beginning graduate students it is designed to accommodate those who require the staff and special facilities of the laboratory in support of their research. Students join staff of the MBL in a variety of studies, participate in courses at the Woods Hole Oceanographic Institution, the Boston University Marine Program, and in regularly scheduled seminars with staff of The Ecosystems Center and others at the MBL.

The Staff

George M. Woodwell, director, The Ecosystems Center
Daniel B. Botkin, associate scientist
Charles A. S. Hall, assistant scientist
John E. Hobbie, senior scientist
Jerry M. Melillo, assistant scientist
Bruce J. Peterson, assistant scientist

THE LABORATORY STAFF

(Including all whose services began or ended during the year)

Homer P. Smith, *General Manager*
Marie B. Abbott, *Curator, Gray Museum*
Lucena J. Barth, *Director of Admissions*
Francis P. Bowles, *Coordinator of Research Services*
Teresa M. Braun, *Assistant Editor, The Biological Bulletin*
Edward G. Casey, *Controller*
Jane Fessenden, *Librarian*
A. Robert Gunning, *Superintendent, Buildings and Grounds*
Lewis M. Lawday, *Assistant Manager, Supply Department*
Anne C. Maher, *Public Relations Officer*
Morton D. Maser, *Coordinator of Continuing Education*
Wesley N. Tiffney, *Curator, Gray Museum*
John J. Valois, *Manager, Supply Department*
George M. Woodwell, *Director, The Ecosystems Center*

EDUCATION OFFICE

Ester M. Browne

DIRECTOR'S OFFICE

Maureen M. Morris

GENERAL MANAGER'S OFFICE

Florence S. Butz
Elaine C. Crocker
Agnes L. Geggatt

PUBLIC RELATIONS OFFICE

Fern P. Callis

CONTROLLER'S OFFICE

Ruth B. Campbell
Doris C. Davis
Nancy L. Ellis
Joan E. Howard
Lorraine A. Ruddick
Anne S. Williams

LIBRARY

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Rebecca J. Bailey
Nancy M. Capuano
Lynne A. Doelling
David J. Fitzgerald
Charlotte F. Goudreau
Joan H. Grice
E. Lenora Joseph
Holly E. Karalekas
Theresa K. McKee
M. Ann White

BUILDINGS AND GROUNDS

Richard Audette
Lee E. Bourgoin
Glenn R. Enos
Charles K. Fuglister
Elizabeth J. Geggatt
Richard E. Geggatt, Jr.
Robert L. Grosch
Marcia Josephson

Thomas N. Kleindinst
Elizabeth Kuil
Donald B. Lehy
Ralph H. Lewis
Sofiea Lewis
William M. Lochhead
Daniel Loewus
Richard A. Lovering
Alan G. Lunn
John B. MacLeod
John E. Maurer
Stephen A. Mills
Simone St. Jean
Glenn I. Shear
Gilbert F. Silvia
Christopher A. Stone
Frederick E. Thrasher
Frederick E. Ward
Ralph D. Whitman
William Whittaker

DEPARTMENT OF RESEARCH SERVICES

Thomas R. Anthony
Franklin D. Barnes
John S. Barnes
Carol E. Bloom
Julie A. Caissie
M. Gale Clark
Linda M. Golder
Robert J. Golder
Kathryn L. Helfrich
Andrew D. Hodgdon
David Juers
Lowell V. Martin
Joan A. Peters-Gilmartin
Frank E. Sylvia

SUPPLY DEPARTMENT

Edward G. Enos, Jr.
Joyce B. Enos
Robert M. Hebden
Mark W. Munson
John H. Ryther, Jr.
Eugene A. Tassinari
Bruno F. Trapasso
John M. Varao

2. Investigators; Research Fellowships; Students

Independent Investigators, 1977

Adelman, William J., Jr., Chief, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Aizu, Seiei, Postdoctoral Fellow, CMDNJ, Rutgers University Medical School
Akaike, Tadashi, Visiting Associate, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Alkon, Daniel L., Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Armstrong, Clay M., Professor of Physiology, University of Pennsylvania
Armstrong, Peter B., Associate Professor of Zoology, University of California
Arnold, John M., Professor of Cytology, University of Hawaii
Atwood, H. L., Professor, University of Toronto
Baldwin, Thomas O., Assistant Professor, University of Illinois
Ball, Eric G., Independent Investigator, Marine Biological Laboratory
Barlow, Robert B., Jr., Professor, Syracuse University
Barrett, Martha Barcalow, Instructor, University of Colorado at Denver
Battelle, Barbara-Anne, Research Fellow, Harvard Medical School
Bauer, G. Eric, Professor of Anatomy, University of Minnesota
Baumgold, Jesse, Staff Fellow, NIMH, National Institutes of Health
Beaugé, Luis A., Associate Professor of Biophysics, University of Maryland School of Medicine
Bennett, Michael V. L., Professor of Neuroscience, Director, Division of Cellular Neurobiology, Albert Einstein College of Medicine of Yeshiva University
Bernard, Gary D., Associate Professor, Yale University
Bezanilla, Francisco, Visiting Lecturer, University of Pennsylvania
Bishop, Stephen H., Assistant Professor of Biochemistry, Baylor College of Medicine

Borgese, Thomas A., Associate Professor of Biology, Herbert H. Lehman College, The City University of New York
Brinley, F. J., Jr., Professor of Biology, University of Maryland
Brodwick, Malcolm S., Assistant Professor, University of Texas Medical Branch
Brown, Jay C., Associate Professor, University of Virginia School of Medicine
Brown, Joel E., Professor of Physiology and Biophysics, State University of New York at Stony Brook
Burdick, Carolyn J., Associate Professor of Biology, Brooklyn College, The City University of New York
Burger, Max M., Chairman of the Biocenter, University of Basel, Switzerland
Burkart, Werner R., Scientific Assistant, Biozentrum, University of Basel, Switzerland
Chang, Donald C., Assistant Professor, Baylor College of Medicine
Chappell, Richard L., Associate Professor, Hunter College, The City University of New York
Charlton, M. P., Postdoctoral Fellow, University of Toronto
Christopher, Alan R., Research Associate, University of Virginia
Claude, Philippa, Assistant Scientist, University of Wisconsin
Cohen, Fredric S., Research Fellow, Albert Einstein College of Medicine
Cohen, Jonathan B., Assistant Professor of Pharmacology, Harvard Medical School
Cohen, Lawrence B., Associate Professor, Yale University School of Medicine
Cohen, Leonard H., Senior Member, Institute for Cancer Research
Cohen, Stanley, Professor of Biochemisty, Vanderbilt University
Cohen, William D., Associate Professor of Biological Sciences, Hunter College, The City University of New York
Cole, Kenneth S., Senior Scientist, National Institutes of Health
Cooperstein, Sherwin J., Professor of Anatomy, University of Connecticut
Crow, Terry J., Postdoctoral Fellow, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
DeFelice, Louis J., Associate Professor, Emory University
de Groof, Robert C., Instructor, Thomas Jefferson University
Dentler, William L., Assistant Professor, University of Kansas
deTerra, Noel, Research Associate Professor, Hahnemann Medical College
De Weer, Paul, Associate Professor of Physiology and Biophysics, Washington University School of Medicine
DiPolo, Reinaldo, Associate Investigator, Instituto Venezolano de Investigaciones Cientificas, Venezuela
Dowling, John E., Professor of Biology, Harvard University
DuBois, Arthur B., Director, John B. Pierce Foundation Laboratory
Dunham, Philip B., Professor of Biology, Syracuse University
Eaton, Douglas C., Assistant Professor of Physiology and Biophysics, University of Texas Medical Branch
Eberhard, Anatol, Associate Professor of Chemistry, Ithaca College
Eckberg, William R., Assistant Professor, Howard University
Edds, Kenneth T., Assistant Scientist, Marine Biological Laboratory
Ehinger, Berndt E. J., Professor, University of Lund, Sweden
Ehrenstein, Gerald, Research Physicist, National Institutes of Health
Ellison, Rebecca P., Postdoctoral Fellow, The Population Council, The Rockefeller University
Epel, David, Professor, University of California, San Diego
Ettienne, Earl M., Associate Professor of Physiology, University of Massachusetts Medical School
Farmanfarmaian, A., Professor of Physiology, Rutgers–The State University of New Jersey
Finkelstein, Morris C., Associate Research Scientist, New York University Medical Center
Fiore, James, Associate Professor of Biology, Suffolk University
Fischbach, Gerald, Associate Professor of Pharmacology, Harvard Medical School
Fischman, Donald A., Professor and Chairman, Department of Anatomy and Cell Biology, State University of New York, Downstate Medical Center
Fishman, George S., Professor of Operations Research and Systems Analysis, University of North Carolina at Chapel Hill

Fishman, Harvey M., Associate Professor of Physiology and Biophysics, University of Texas Medical Branch
Fohlmeister, Jurgen, Lecturer, University of Minnesota
Fletcher, Donald James, Postdoctoral Research Investigator, Emory University
Freeman, Gary, Associate Professor, University of Texas, Austin
French, Robert J., Visiting Associate, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Furshpan, Edwin J., Professor of Neurobiology, Harvard Medical School
Gainer, Harold, Head, Section of Functional Neurochemistry, National Institutes of Health
Gibson, Jane, Associate Professor of Biochemistry, Cornell University
Gilbert, Daniel L., Research Physiologist, NINCDS, National Institutes of Health
Goldman, David E., Guest Worker, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Goldsmith, Paul K., Biologist, NIAMD, National Institutes of Health
Goodman, Lesley Jean, Senior Lecturer, Queen Mary College, London
Greenberg, Michael John, Professor, Florida State University
Grinvald, Amiram, Postdoctoral Associate, Yale University School of Medicine
Grosch, Daniel S., Professor of Genetics, North Carolina State University
Gross, Cordell E., Chief Resident in Neurosurgury, State University of New York, Upstate Medical Center
Grossman, Albert, Professor, New York University School of Medicine
Grossman, Yoram, Visiting Fellow, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Guttman, Rita, Professor of Biology, Brooklyn College, The City University of New York
Hall, B. D., Professor of Genetics, University of Washington
Halvorson, Harlyn O., Director, Rosenstiel Basic Medical Sciences Research Center, and Professor of Biology, Brandeis University
Hanass, Menachem, Visiting Fellow, Laboratory of Neurophysiology, NINCDS, National Institutes of Health
Hanna, Robert B., Instructor, Albert Einstein College of Medicine
Harding, Clifford V., Professor and Director of Research, Kresge Eye Institute, Wayne State University
Haschemeyer, Audrey E. V., Professor of Biology and Biochemistry, Hunter College, The City University of New York
Hastings, John Woodland, Professor of Biology, Harvard University
Heldman, Elihu, Biochemist, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Henderson, Mary Ann R., Associate Professor, Rollings College
Heuser, John, Associate Professor, University of California, San Francisco
Highstein, Stephen M., Associate Professor of Neuroscience, Albert Einstein College of Medicine
Hill, Robert B., Professor of Zoology, University of Rhode Island
Hoffmann, Richard J., Assistant Professor, University of Pittsburgh
Horn, Richard, Postdoctoral Fellow, University of Texas Medical Branch
Hoskin, Francis C. G., Professor of Biology, Illinois Institute of Technology
Humphreys, Tom, Associate Professor, University of Hawaii
Hungate, Robert E., Professor Emeritus of Bacteriology, University of California, Davis
Hunt, R. T., Research Fellow, University of Cambridge, England
Hunt, Richard Kevin, Assistant Professor, The Johns Hopkins University
Inoue, Sadayuki, Assistant Professor, McGill University, Canada
Inoué, Shinya, Professor of Biology, Director, Program in Biophysical Cytology, University of Pennsylvania
Isenberg, Irvin, Professor, Oregon State University
Jung, Walter K., Research Fellow in Pathology, Harvard Medical School, Massachusetts General Hospital, Shriners Burns Institute
Kabat-Zinn, Jonathan, Postdoctoral Research Associate, University of Massachusetts Medical School
Kaiserman-Abramof, Ita R., Associate Professor, Case Western Reserve University

Kaminer, Benjamin, Professor and Chairman, Boston University School of Medicine
Kamino, Kohtaro, Postdoctoral Associate, Yale University School of Medicine
Kaplan, Ehud, Assistant Professor, The Rockefeller University
Kem, William Reade, Assistant Professor, University of Florida College of Medicine
Kennedy, William R., Professor of Neurology, University of Minnesota
Keosian, John, Professor Emeritus, Rutgers–The State University of New Jersey
Keynan, Alex, Vice President, The Hebrew University, Israel
Kirsch, Glenn E., Postdoctoral Fellow, Northwestern University
Kline, Richard Paul, Research Fellow, Harvard University
Klotz, Lynn C., Instructor, Physiology Course, Marine Biological Laboratory
Kogut, Margot, Lecturer, King's College, London, England
Koide, Samuel S., Senior Scientist, The Population Council, The Rockefeller University
Konishi, Shiro, Research Fellow, Harvard Medical School
Kravitz, Edward A., Professor of Neurobiology, Harvard Medical School
Kriebel, Mahlon E., Associate Professor of Medicine, State University of New York, Upstate Medical Center
deKruyf, H. A. M., Assistant Director, Principal Research Officer, Caribbean Marine Biological Institute, Curacao, Netherlands Antilles
Kuffler, Stephen W., John Franklin Enders University Professor, Harvard Medical School
Kuhns, William J., Associate Professor of Pathology, New York University School of Medicine
Kurek, Michael P., Postdoctoral Fellow, Case Western Reserve University
Landowne, David, Associate Professor, University of Miami
Lasek, Raymond J., Associate Professor, Case Western Reserve University
Lash, James W., Professor of Anatomy, University of Pennsylvania
Laufer, Hans, Professor of Biology, University of Connecticut
Leadbetter, E. R., Professor of Biology, Amherst College
Lederhendler, I. Izsa, Visiting Fellow, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Lee, John J., Professor of Biology, City College, The City University of New York
Lester, Roger, Professor and Chief of Gastroenterology, University of Pittsburgh School of Medicine
Levin, Jack, Associate Professor of Medicine, The Johns Hopkins University School of Medicine
Levinthal, Cyrus, Professor of Biology, Columbia University
Levitan, Herbert, Associate Professor, University of Maryland
Lewis, T. J., Professor, University of North Wales, Wales
Lipicky, Raymond John, Professor of Pharmacology, Professor of Medicine and Director of Clinical Pharmacy, University of Cincinnati
Liuzzi, Anthony, Associate Professor, University of Lowell
Livingstone, Margaret S., M.D. Ph.D. Student, Harvard Medical School
Llinas, R., Professor and Chairman of Department, New York University Medical Center
Lockau, Wolfgang, Research Associate, Michigan State University
Loewenstein, W. R., Professor and Chairman, Department of Physiology and Biophysics, University of Miami School of Medicine
Lorand, L., Professor of Biochemistry and Molecular Biology, Northwestern University
Low, Philpip Anson, Mayo Scholar, Mayo Clinic
MacLeish, Peter R., Postdoctoral Fellow, Harvard Medical School
Mantel, Linda Habas, Associate Professor of Biology, City College, The City University of New York
Maruo, Takeshi, Postdoctoral Fellow, The Population Council, The Rockefeller University
Mastroianni, Luigi, Jr., Professor and Chairman, Department of Obstetrics and Gynecology, University of Pennsylvania, School of Medicine
Mathews, Rita W., Senior Research Associate, Hunter College, The City University of New York
Matsumura, Fumio, Professor, University of Wisconsin
Mauro, Alexander, Professor of Biophysics, The Rockefeller University
Mauzerall, David, Professor, The Rockefeller University
Metuzals, J., Professor, Director of the Electron Microscopy Unit, University of Ottawa, Canada

Metz, Charles B., Professor, University of Miami
Miller, Richard S., Professor, Yale University
Mitchell, Ralph, Gordon McKay Professor of Applied Biology, Harvard University
Moore, John W., Professor of Physiology, Duke University
Moore, Lee, Professor, University of Texas Medical Branch
Mooseker, Mark S., Research Fellow, Harvard Medical School
Myles, Christina J., Assistant Professor of Life Science, Manchester Community College
Nagel, Ronald L., Associate Professor, Albert Einstein College of Medicine
Nakajima, Shigehiro, Professor, Purdue University
Nakajima, Yasuko, Professor, Purdue University
Narahashi, Toshio, Professor and Chairman, Northwestern University
Newrock, Kenneth, Postdoctoral Research Associate, Institute for Cancer Research, Fox Chase Cancer Center
Nicoli, Miriam Z., NIH Postdoctoral Fellow, University of Illinois
Nielsen, Jennifer B. K., Senior Research Associate, Hunter College, The City University of New York
Noe, Bryan D., Assistant Professor of Anatomy, Emory University
Oertel, Donata, Postdoctoral Fellow, Harvard University Medical School
O'Lague, Paul H., Assistant Professor of Biology, University of California, Los Angeles
Ornberg, Richard L., Research Assistant, Neurophysiologist, Pennsylvania State University and National Institutes of Health
Oschman, James L., Investigator, Marine Biological Laboratory
Oxford, Gerry S., Assistant Professor of Physiology, University of North Carolina at Chapel Hill
Pant, Harish C., Senior Staff Fellow, NIMH, National Institutes of Health
Pappas, George Demetrios, Professor of Anatomy and Neuroscience, Albert Einstein College of Medicine
Pardy, Rosevelt L., Assistant Dean, Biological Sciences, University of California, Irvine
Parmentier, James L., Postdoctoral Fellow, Duke University
Person, Philip, Medical Investigator, Veterans Administration Hospital, Brooklyn
Pethig, R., Lecturer, University of North Wales, Wales
Pierce, Sidney K., Associate Professor, University of Maryland
Pollard, Harvey B., Senior Investigator and Medical Officer, National Institutes of Health
Potter, David D., Professor of Neurobiology, Harvard Medical School
Poussart, Denis, Associate Professor, Université Laval, Canada
Prendergast, Robert A., Associate Professor of Ophthalmology and Pathology, The Johns Hopkins University
Prior, David J., Assistant Professor, University of Kentucky
Prusch, Robert D., Assistant Professor of Biomedical Sciences, Brown University
Pumplin, David W., IPA Biologist, LNNS, NINCDS, National Institutes of Health
Quigley, James P., Assistant Professor of Microbiology and Immunology, State University of New York, Downstate Medical Center
Ramon, Fidel, Assistant Professor, Duke University
Reese, Thomas, Head, Section of Functional Neuroanatomy, LNNS, NINCDS, National Institutes of Health
Reynolds, George T., Professor of Physics, Princeton University
Rheuben, Mary B., Assistant Professor, Pennsylvania State University
Rice, Robert V., Professor and Head, Department of Biological Sciences, Mellon Institute of Science, Carnegie-Mellon University
Rickles, Frederick R., Associate Professor of Medicine, Hematologist, Veteran Administration Hospital and University of Connecticut Health Center
Ripps, Harris, Professor of Ophthalmology and Physiology, New York University School of Medicine
Rose, Birgit, Assistant Professor, University of Miami School of Medicine
Rosen, Jeffrey M., Assistant Professor of Cell Biology, Baylor College of Medicine
Rosenbaum, Joel L., Associate Professor of Biology, Yale University
Rushforth, Norman B., Professor and Chairman, Department of Biology, Case Western Reserve University
Russell, John M., Assistant Professor, University of Texas Medical Branch

Russell-Hunter, W. D., Professor of Zoology, Syracuse University
Saffo, Mary Beth, Miller Postdoctoral Fellow, University of California, Berkeley
Salmon, Edward D., Assistant Professor of Zoology, University of North Carolina at Chapel Hill
Scarpa, Antonio, Associate Professor, University of Pennsylvania
Schairer, John Otto, M.D., Ph.D. Student, Albert Einstein College of Medicine
Schein, Stanley Jay, Postdoctoral Fellow, Massachusetts Institute of Technology
Schiff, Jerome A., Professor of Biology and Director of the Institute for Photobiology of Cells and Organelles, Brandeis University
Schuetz, Allen W., Professor, The Johns Hopkins University
Segal, Sheldon J., Vice President and Director, Biomedical Division, The Population Council, The Rockefeller University
Seyama, Issei, Postdoctoral Research Associate, Northwestern University
Shecket, Gordon, Graduate Student, Case Western Reserve University
Shick, J. Malcolm, Assistant Professor of Zoology, University of Maine at Orono
Shoukimas, Jonathan J., IPA Fellow, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health
Shrivastav, Brij B., Medical Research Assistant Professor, Duke University
Simmons, Robert M., Lecturer, University College, London, England
Simon, Robert D., Assistant Professor, University of Rochester
Singer, Robert H., Assistant Professor of Anatomy, University of Massachusetts Medical School
Smith, Michael A. K., Research Associate, Hunter College, The City University of New York
Sobel, Matthew J., Associate Professor, Yale University
Spiegel, Evelyn, Research Associate, Dartmouth College
Spiegel, Melvin, Professor of Biology, Dartmouth College
Spira, Micha E., Albert Einstein College of Medicine
Spray, David, Research Fellow, Albert Einstein College of Medicine
Srinivasan, Mandyam V., Research Staff Scientist, Yale University
Stavenga, Doekele Gerben, Wetenschappelijkmedewerker, Department of Biophysics, Rijksuniversiteit Groningen, The Netherlands
Steinacker, Antoinette, Instructor, CMDNJ, Rutgers University Medical School
Stephens, R. E., Associate Professor, Brandeis University
Stetten, Marjorie R., Biochemist, NIAMDD, National Institutes of Health
Stickgold, Robert, Research Fellow, Harvard Medical School
Stokes, Darrell R., Assistant Professor, Emory University
Storti, Robert V., Visiting Scientist, Massachusetts Institute of Technology
Stracher, Alfred, Professor and Chairman, Department of Biochemistry, State University of New York, Downstate Medical Center
Strauss, Jerome F., III, Associate, University of Pennsylvania School of Medicine
Stretton, A. O. W., Professor of Zoology, University of Wisconsin
Stuart, Ann Elizabeth, Assistant Professor, Harvard Medical School
Stunkard, Horace W., Research Associate, American Museum of Natural History
Sugimori, Mutsuyuki, Research Scientist, New York University
Susswein, Abraham J., Albert Einstein College of Medicine, Yeshiva University
Szamier, R. Bruce, Assistant Professor, Harvard Medical School
Szent-Gyorgyi, Andrew G., Chairman, Department of Biology, Brandeis University
Szentkiralyi-Szent-Gyorgyi, Eva M., Research Associate, Brandeis University
Takashima, Shiro, Professor, Graduate Group Chairman, University of Pennsylvania
Tasaki, Ichiji, Chief, Laboratory of Neurobiology, NIMH, National Institutes of Health
Taylor, Robert E., Supervisory Research Physiologist, NINCDS, National Institutes of Health
Taylor, Stuart R., Consultant in Pharmacology, Mayo Clinic Foundation
Terakawa, Susumu, Visiting Associate, NIMH, National Institutes of Health
Tifferet, Teresa, Research Associate, University of Maryland School of Medicine
Tilney, Lewis C., Associate Professor of Biology, University of Pennsylvania
Tipper, Donald J., Chairman, University of Massachusetts Medical School
Troll, Walter, Professor of Environmental Medicine, New York University Medical Center
Troxler, Robert F., Associate Professor of Biochemistry, Boston University School of Medicine
Tweedell, Kenyon S., Professor, University of Notre Dame

Van Holde, K. E., Professor of Biophysics, Oregon State University
Van Raalte, Charlene, Course Coordinator, Dalhousie University, Nova Scotia
vander Westhuyzen, Deneys R., USPHS International Postdoctoral Fellow, State University of New York, Downstate Medical Center
Vincent, Walter S., Professor of Cell Biology, University of Delaware
Wagner, Richard W., Postdoctoral Fellow, University of Miami
Wall, Betty J., Investigator, Marine Biological Laboratory
Waxman, Lloyd H., Postdoctoral Fellow, University of California, Davis
Weber, Annemarie, Professor, University of Pennsylvania
Weber, Michel, Research Associate, Harvard Medical School
Weinstein, Paul P., Professor, University of Notre Dame
Weissmann, Gerald, Professor of Medicine, Director, Division of Rheumatology, New York University School of Medicine
Wells, Jay B., Research Physiologist, NINCDS, National Institutes of Health
Westerfield, Monte, Postdoctoral Fellow, Duke University
Whittaker, J. Richard, Associate Member, Wistar Institute
Williams, Janet M., Investigator, Marine Biological Laboratory
Williams, Theodore P., Professor of Biological Sciences, Florida State University
Wilson, Darcy B., Professor, University of Pennsylvania, School of Medicine
Wolf, Don P., Assistant Professor, University of Pennsylvania, School of Medicine
Wu, Chau H., Assistant Professor of Pharmacology, Northwestern University
Yeh, J. Z., Assistant Professor, Northwestern University
Zigman, Seymour, Associate Professor of Ophthalmology and Biochemisty, University of Rochester School of Medicine and Dentistry
Zimmerberg, Joshua, Medical Scientist Trainee, Albert Einstein College of Medicine
Zimmerman, Morris, Senior Research Fellow, Merck Institute for Therapeutic Research

Lillie Fellows, 1977

Okada, Tokindo, Institute of Biophysics, Kyoto, Japan
Warner, Ann, Senior Lecturer in Anatomy and Embryology, University College, London, England

Alexander Forbes Lecturers, 1977

Asher, Phillippe, Laboratoire de Neurologie, Paris, France
Kehoe, JacSue, Lecturer, Laboratoire de Neurologie, Paris, France

Rand Fellow, 1977

Larson, Helge, Professor of Biochemistry, Norwegian Institute of Technology, Norway

Junior Rand Fellow, 1977

Matsuhashi, Sachiko, Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan

Grass Fellows, 1977

Frazier, Donald T., Senior Fellow, Professor, University of Kentucky, School of Medicine
Chamberlain, Steven, Graduate Student, Institute for Sensory Research, Syracuse University
Elliott, Ellen, Research Fellow, Department of Zoology, University of Maryland
Fricke, Russell A., Postdoctoral Fellow, Department of Biological Sciences, Stanford University
Friedman, Richard N., Graduate Student, Department of Physiology, Temple University
Fuchs, Paul A., Fellow, Department of Biological Sciences, Stanford University
Impelman, Dianne, Research Associate in Ophthalmology, Duke University Eye Center
Kennedy, Brian G., Graduate Student, Washington University School of Medicine
Meiss, Dennis E., Research Postdoctorate, Scarborough College, University of Toronto
Pierson, Martha, Researcher, Defense Nuclear Agency, AFFRI

Russell, David F., Postdoctoral Research Associate, Biology Department, University of California, San Diego
Schmidt, Jeffrey A., Grass Fellow, Department of Biology, University of California

Macy Scholars, 1977

Adams, James A., Assistant Professor of Biology, Tennessee State University
Armstrong, Earlene, Assistant Professor of Entomology, University of Maryland
Hill, Anita V., Assistant Professor of Biology, Grambling State University
Howze, Gwendolyn, Assistant Professor, Texas Southern University
Langford, George M., Assistant Professor, Howard University
Sutton, William W., Assistant Professor, Dillard University

Junior Macy Scholar, 1977

Lyn-Cook, L. E., Howard University

Summer Research Scholarships, 1977
(Steps Toward Independence)

Armett-Kibel, Christine, Assistant Professor of Biology, University of Massachusetts
Burns, Roy G., Postdoctoral Research Fellow, University of Edinburgh, Scotland
Carroll, Edward J., Jr., Assistant Professor of Biology, University of California
Linck, Richard W., Assistant Professor, Harvard Medical School
Meinertzhagen, I. A., Assistant Professor, Dalhousie University, Nova Scotia, Canada
Murphy, Douglas B., Assistant Professor, Kansas State University
Salzberg, Brian M., Assistant Professor of Physiology, University of Pennsylvania
Young, Lily Y., Assistant Professor, Stanford University

Research Assistants, 1977

Adair, Wyn Steven, Wesleyan University
Anderson, James M., Harvard University
Antonellis, Blenda, University of Rochester
Armstrong, Richard A.
Augustine, George J., Jr., University of Maryland
Barkalow, Derek T., Rutgers—The State University of New Jersey
Barkalow, Ruth A., Clark University
Barnes, Susan J., Columbia University
Battey, James Frank, University of Michigan
Beach, D. H., University of Miami Medical School
Belkin, Michael, University of Connecticut Health Center
Bennett, Holly Vander Laan
Bennetzen, Jeffrey L., University of Washington
Binder, Robert, Cornell University
Bosler, Robert B., Harvard Medical School
Caffrey, John M., Duke University
Caldwell, Kathy
Cappell, Mitchell S., Albert Einstein College of Medicine
Cartabuke, Richard, Case Western Reserve University
Carter, Margaret M., University of Delaware
Cartwright, Joiner, Jr., University of Hawaii
Cassidy, F. P., Jr., University of Pittsburgh
Cheney, Clarissa M., University of Pennsylvania
Clark, John M., University of Wisconsin
Cohen, Dan, University of Cincinnati College of Medicine
Colucci, Barbara Ann, Lehman College, The City University of New York
Cooperstein, Larry, University of Rochester

Csernansky, John, New York University School of Medicine
Dodge, F. A., The Rockefeller University
DuBois, Brooks, Case Western Reserve University
DuBois, Roland C., Carl Zeiss Corporation
Duh, Quan-Yang
Lanyon-Duncan, Cynthia H.
Eagle, Jane, University of Connecticut Health Center
Ebihara, Lisa, Duke University Medical Center
Edgington, Duane R., Harvard Medical School
Eisen, Andrew, University of Pennsylvania School of Medicine
Feldman, Lance, University of Texas Medical Branch
Ferraris, Joan, Marine Science Institute
Fetterer, Raymond H., Michigan State University
Fine, Alan, University of Pennsylvania
Finger, Thomas, Washington University School of Medicine
Finkel, Toren, University of California, Riverside
Fitt, Bill, University of California, Santa Barbara
Fox, Sarah Jane, Radcliffe College
Gall, Barbara, University of Colorado
Gallager, Scott M.
Galvin, Patric Neal, University of Colorado, Denver
Gerber, Randall G., University of Hawaii
Glicksman, Marcie, Brown University
Glogau, Alexander Ives, City College, The City University of New York
Gordon, Gerald, University of Pennsylvania
Gravis, Charles M., State University of New York at Buffalo
Grip, Jeremy, Illinois Institute of Technology
Gross, Robin, Simmons College
Hacopian, Vachik, University of Massachusetts, Amherst
Hankins, Carol A., Simmons College
Harary, Howard H., Harvard University
Harris, Andrew L., Albert Einstein College of Medicine
Hayes, William Par, University of Rhode Island
Hinton, Joanna P., Ithaca College
Hopkins, Roswitha G., Oregon State University
Hosmer, Hillary A., University of California, Irvine
Hunter, Ann W., Emory University
Hurst, Terry W., University of Texas Medical Branch
Huse, William D., Albert Einstein College of Medicine
Huttner, Susanne, University of California, Los Angeles
Howard, Jacobs, University of Hawaii
Jaeger, Richard F., Hunter College, The City University of New York
Jaslove, Stewart, Duke University
Johnson, Harold E., Jr., University of Pennsylvania
Johnston, Michael F., Duke University
Josephs, Nathan M.
Josephson, Beth
Kagan, Bruce, Albert Einstein College of Medicine
Kenny, Carol, University of Rochester
Kiehart, Daniel P., University of Pennsylvania
LaFratta, James M., Harvard Medical School
Landesman, Jeffrey, City College, The City University of New York
Landzberg, Joel S., Columbia University
Laufer, Marc Reed
Lesher, Sarah, Yale University
Lifset, Reid, Swarthmore College
Liu, Chi-Being, Washington University
Lowenhaupt, Manuel, Massachusetts Institute of Technology

Lyn-Cook, Lascelles E., Howard University
Massell, David, Buxton School
Matheke, Mary Lou, Columbia University
McCarthy, Robert A., Dartmouth College
Mir, Francoise, University of California, San Diego
Mishkind, Michael, State University of New York at Stony Brook
Muratore, John F., Yale University
Nelson, Mark T., Washington University
Nemhauser, Iris, Hunter College, The City University of New York
Nicklas, Nancy Lynn, University of Pittsburgh
Otto, James, University of Maryland
Perry, Gavin, Washington University
Pratt, Melanie M., Brandeis University
Puccia, Charles J., The Ecosystems Center, Marine Biological Laboratory
Reiss, Paul, University of Maryland
Richardson, Ann L., Louisiana State University
Riives, Mai, Dalhousie University, Nova Scotia
Robertson, Lola, American Museum of Natural History
Robinson, Mark V, Rutgers—The State University of New Jersey
Rosman, Gary, Emory University
Samson, Douglas A., The Johns Hopkins School of Hygiene and Public Health
Schatz, Scott, University of Rhode Island
Schlup, Verena, Biocenter, University of Basel, Switzerland
Schmeidler, Katherine T., Case Western Reserve University
She, Joseph, University of Toronto
Siegel, Ruth, Harvard Medical School
Siegel, Sharon, Albert Einstein College of Medicine
Smith, S. Gregory, Kresge Eye Institute, Wayne State University
Smith, Tonicia M.
Spanjers, Mary Anna, Tulane University
Stich, Thomas J., University of Maryland
Suh, Kyungsun, University of Texas, Austin
Swenson, R. P., Jr., Duke University
Tatchell, Kelly G., Oregon State University
Techner, Lee, Syracuse University
Valanti, Iris
Von Hippel, David F.
Wachtel, Ruth E., Northwestern University
Wadsworth, Patricia, St. Lawrence University
Walker, Jeff W., University of California, Berkeley
Walton, Kerry, New York University
Wodlinger, Harold M., University of Toronto
Wolf, Brian Edmund, Amherst College
Wong, Winston C., Baylor College of Medicine
Wright, Nancy Joan, Duke University
Yantorno, Robert E., University of Pennsylvania
Yonemoto, Wes, University of Hawaii
Young, Steven H., Duke University
Yulo, Teresa, University of Rochester
Zakevicius, Jane M., New York University School of Medicine
Zecevic, Dejan, University of Maryland
Zigman, Sheree, University of Rochester
Zuck, Robin, Ithaca College
Zukowski, Anthony J., Marietta College

Library Readers, 1977

Adelberg, Edward A., Professor of Human Genetics, Yale University
Allen, Nina Stromgren, Assistant Professor, Dartmouth College

ALLEN, ROBERT DAY, Professor and Chairman, Dartmouth College
ANDERSON, EVERETT, Professor of Anatomy and Associate Director, Laboratory of Human Reproduction and Reproductive Biology, Harvard University Medical School
BARRIOS, ROBERTO, Pathologist, Universidad Nacional Autonoma de Mexico, Mexico
BELL, EUGENE, Professor of Biology, Massachusetts Institute of Technology
BUCK, JOHN B., Chief, Section on Comparative Physiology, National Institutes of Health
CARLSON, FRANCIS D., Professor, The Johns Hopkins University
CARRIERE, RITA, Assistant Professor, State University of New York, Downstate Medical Center
CHILD, FRANK M., Professor and Chairman of Biology, Trinity College
CLIFFORD, SISTER ADELE, Professor of Biology, College of Mount St. Joseph
COLWIN, ARTHUR, Adjunct Professor, University of Miami
CLUSIN, WILLIAM, Resident in Medicine, Stanford University
COPELAND, DONALD EUGENE, Professor of Biology, Tulane University
CORNWELL, ANNE CHRISTAKE, Assistant Professor of Ophthalmology, Montefiore Hospital and Medical Center
DAVIS, BERNARD D., Professor, Harvard Medical School
DEGROOT, NATHAN, Professor, Hebrew University, Israel
DETOLEDO, LEYLA, Associate Professor, Rush Medical College and University of Toronto
DETTBARN, WOLF-D., Professor of Pharmacology, Vanderbilt University School of Medicine
DIXON, KEITH E., Senior Lecturer in Biology, Flinders University of South Australia
EDDS, LOUISE, Instructor, Neuropathology, Harvard Medical School and Children's Hospital Medical Center, Boston
EDER, HOWARD A., Professor of Medicine, Albert Einstein College of Medicine
FRANZINI-ARMSTRONG, CLARA, Associate Professor in Anatomy, University of Pennsylvania
FUSSELL, CATHERINE P., Associate Professor, Pennsylvania State University
GELPERIN, ALAN, Associate Professor of Biology, Princeton University
GOLDMAN, ROBERT D., Associate Professor of Biological Sciences, Carnegie-Mellon University
GOLDSTEIN, MOISE H., Professor, The Johns Hopkins University
GORDON, WILLIAM E., Postdoctoral Fellow, Cold Spring Harbor Laboratory
GOULD, STEPHEN JAY, Professor of Geology, Harvard University
GRANT, PHILIP, Professor of Biology, University of Oregon
GUNSALUS, I. C., Professor of Biochemistry, University of Illinois
HINSCH, GERTRUDE W., Associate Professor, University of South Florida
ILAN, JOSEPH, Professor, Case Western Reserve University
IMBERT, MICHEL, Deputy Director, College de France, France
ISSELBACHER, KURT J., Mallinckrodt Professor of Medicine, Chief, Gastrointestinal Unit, Harvard Medical School and Massachusetts General Hospital
JOHNSON, W. CURTIS, Associate Professor of Biophysics, Oregon State University
JOHNSON-MULLER, BARBARA PAULE, Postdoctoral Research Fellow in Medicine, Harvard Medical School
KALTENBACH, JANE C., Professor of Biological Sciences, Mount Holyoke College
KARUSH, FRED, Professor of Microbiology, University of Pennsylvania
KIRSCHENBAUM, DONALD M., Associate Professor, College of Medicine, State University of New York, Downstate Medical Center
KLEIN, MORTON, Professor of Microbiology, Temple University
KRUPA, PAUL L., Professor of Biology, City College, The City University of New York
LADERMAN, AIMLEE D., Doctoral Candidate, Teaching Assistant, State University of New York at Binghamton
LAWLESS, SISTER KYRAN JOYCE, Professor of English, Kentucky State University
LEIGHTON, JOSEPH, Professor and Chairman, Department of Pathology, Medical College of Pennsylvania
LEWIS, LARRY M., Research Associate and Assistant Professor, City Univestity of New York, and Millersville State College
LINEAWEAVER, THOMAS H., Author
MARSLAND, DOUGLAS, Research Professor Emeritus, New York University
MIZELL, MERLE, Professor of Biology, Tulane University
MORRELL, FRANK, Professor of Neurology, Rush Medical College
NEWBURY, THOMAS KELLOGG, Assistant Professor

Oosting, Pieter H., Research Scientist, Philips Research Laboratories

Osornio, Alvaro, Pathology Resident at the Cardiology Institute, National University of Mexico

Palmer, John D., Professor, and Chairman Department of Zoology, University of Massachusetts

Pearlman, Alan L., Associate Professor of Physiology and Neurology, Washington University School of Medicine

Plocke, Donald J., S. J., Chairman, Department of Biology, Boston College

Potasek, M. J., Instructor Faculty Member, Princeton University

Potter, H. David, Associate Professor, Indiana University

Przybylski, Ronald J., Associate Professor, Case Western Reserve University

Rosenberg, Evelyn K., Professor of Biology, Jersey City State College

Sager, Ruth, Professor, Sidney Farber Cancer Institute and Harvard Medical School

Saunders, John W., Professor of Biology, State University of New York at Albany

Scott, George T., Professor of Biology, Oberlin College

Shemin, David, Professor and Chairman of Biochemistry and Molecular Biology, Northwestern University

Shepro, David, Professor, Boston University

Sherman, Irwin W., Professor and Chairman, University of California, Riverside

Speck, William T., Director of In-Patient Services, Case Western Reserve University

Spector, Abraham, Professor of Ophthalmic Biochemistry, Columbia University

Staffan, M. E. Magnusson, Atdehings Leder, University of Aarhus, Denmark

Sussman, Maurice, Professor and Chairman, Department of Life Sciences, University of Pittsburgh

Steinhardt, Jacinto, Emeritus Professor of Chemistry, Georgetown University

Tanzer, Marvin L., Professor of Biochemistry, University of Connecticut Health Center

Trinkaus, John Philip, Professor of Biology, Yale University

Wainio, Walter, Professor of Biochemistry, Rutgers—The State University of New Jersey

Walker, Charles A., Dean and Professor, Florida A & M University

Webb, H. Marguerite, Professor of Biological Sciences, Goucher College

Weiss, Leon, Chairman, Department of Animal Biology, Professor of Cell Biology, University of Pennsylvania School of Veterinary Medicine

Wheeler, George E., Professor of Biology, Brooklyn College, The City University of New York

Wilson, Thomas H., Professor of Physiology, Harvard Medical School

Wittenberg, Jonathan B., Professor of Physiology, Albert Einstein College of Medicine

Yntema, Chester L., Professor Emeritus, State University of New York, Upstate Medical Center

Zacks, Sumner Irwin, Pathologist-in-Chief, The Miriam Hospital, and Professor of Pathology, Section Chairman of Pathology, Brown University

Students, 1977

All students listed completed the formal course programs. Asterisk indicates completing post-course research programs.

Summer Programs 1977

ECOLOGY

Adler, Hilary
Barbacci, Elsa
Bransfield, Mark
dePamphilis, Claude
Dionne, Michael
Guzik, Linda
Harries, William
Harvey, Ronald
Janis, Richard
Krezoski, John
Morgan, Hilary
Olson, John
Radomski, William
Robinson, Mary
Robison, Margaret
Sladovich, Hedy
Smith, Patricia
Stout, Heather
Thomason, Philip

EMBRYOLOGY

Anderson, Kenneth
Aune, Thomas
Bader, David
Beach, David
Bean, Charles
Black, Virginia
Cummins, Joseph
Emmerling, Mark
Fraser, Scott
Garling, David
Hoskins, Sally
Jerdan, Janice
Johnston, Randal
Jones, Estralita
Lopo, Alina
Naftel, John
Poole, Thomas
Rho, Jai-Hyon
Robinson, Audrey
Thompson, Holly
Tonelli, Quentin
Tosney, Kathryn
Tucker, Edward
Wiley, Henry
Williams, Edward

EXPERIMENTAL INVERTEBRATE ZOOLOGY

Ben-Eliahu, Maxine
*Beres, Linda
*Birchard, Geoffrey
Brooks, Alfred
*Carbonell Colette
Denys, Charlene
*Dolliver, Elizabeth
Drolet, Lawrence
*Garcia-Arraras, Jose
Gibson, Marcia
Henry, Joseph
Johns, Priscilla
Johnston, James
Kayer, Susan
Kirkpatrick, Steven
Klotz, Alan
Kohn, Barbara
Koumjian, Lauren
Ludel, Jacqueline
Mahaffey, James
Marcus, Paula
*McIlvain, James
*Meyer, Carol
Mitchell, Brian
Monahan, Kevin
Morgan, Charles
Nakashima, Richard
Ortiz-Corps, Edgar
Pierpont, Nina
*Sandridge, Paul
Smullin, David
*Solondz, Leonard
Scott, James D.

EXPERIMENTAL MARINE BOTANY

Burns, Patricia
Davidson, Dionne
Davidson, Eric
*Dwyer, Deborah
*Gates, Cynthia
Harris, Gordon
Kerby, Nigel
*Lane, Melody
Lee, Debbie
Miller, Linda
Price, Ellen
*Schneider, Rebecca
*Scott, Bruce
Scott, Laura
Thorington, Glyne
*Turetsky, Oxana

NEUROBIOLOGY

Aceves-Pina, Efrain
Feinstein, Susan
Harris, Roger
Kass-Simon, Gabriele
Nishi, Rae
Olson, Julie
Peng, Hsiao-ming
St. Marie, Richard
Salpeter, Shelly
Sigworth, Frederick
Stevenson, Bruce
Stieve, Henning

PHYSIOLOGY

*Arisaka, Fumio
Baldwin, Albert
Binder, Michael
*Boothby, Mark
Brenner, Stephen
Bryan, Philip N.
Burks, Douglas
*Caron, Joan
*Cypher, Christopher
Dill, Kenneth
Dillon, Peter
Esmailzadeh, Ardalan
*Frosch, Matthew
Herlands, Louis
*Howe, Christine
*Hurwitz, Julia
Ierardi, Lynne
Johnson-Muller, B.
*Lassar, Andrew
Leipzig, George
*Lerner, Ethan
*Matsudaira, Paul
Matteson, Donald
*Mercurio, Arthur
*Merritt, Margaret
*Ohriner, William
*Porter, Mary
*Price, David H.
Reeve, Carole
Sellers, James
Seminara, Daniela
Setzer, David
Sweetman, Harold
Whittaker, Carla

January Programs 1977

BEHAVIOR

Berry, Ronald R.
Bratton, Bradford O.
Cavanaugh, Colleen M.
Chambers, David B.
Dourdeville, Theodore A.
Frick, Jane A.
Friedlander, Amy I.
Gemperline, Clare H.
Giblin, Anne E.
Greene, Sharon I.
Hanych, David A.
Hickman, Roberta A.
Howes, Brian L.
Hutton, Edward W.
Pappas, Georgia
Perkins, Judith S.
Roberts, Sandra F.
Rose, Kenneth J.
Watkins, Brenda F.
Werme, Christine E.

DEVELOPMENTAL BIOLOGY

Bischoff, Kimberly J.
Dinsmore, Christine G.
Donahue, Robert E.
Ecker, Marilyn M.
Gildea, Janet C.
Harris, Gordon
Hawkins, Willie J.
Henry, Joseph M.
Hunter, Joyce M.
Loftfield, Katherine
Lyndes, Harry E. III
May, Sarah E.
Spanjers, Mary A.
Urushihara, Hideko

ECOLOGY

Beese, Lorena S.
Bouwkamp, Carl A.
Charles, Ann M.
Coney, Debra S.
Haas, Eric N.
Hoar, Peter R.
Hope, Stephen L.
Neilson, Ronald P.
Pearlmutter, Gary
Rosenberg, Andrew A.
Saxe, Michael R.
Shamonsky, Margie
Thoreson, Brian
Turner, K. Alta
Unger, Geri E.
White, Mark N.
Younger, Dana R.

NEUROBIOLOGY

Anderson, Carl B.
Avnet, Mark S.
Bryant, Bruce P.
Burke, Briant E.
Calof, Anne Leighton
Camelio, Elsie
Cleveland, Mark V. B.
Cole, James J.
Jacobson, Myrna E.
Lederhendler, Israel I.
Maxwell, John A.
Mellin, Theodore N.
Roy, Bonnitta
Sahley, Christie L.
Sartor, Alton Oliver
Smith, Ronald E.
Stetten, George D.
Strassman, Jeffrey C.
Vitale, Anthony J.
Wang, Ching Chung
Whetzel, Thomas P.
Wilson, Donella J.

BOSTON UNIVERSITY MARINE PROGRAM

Ashkenas, Linda
Botero, Leonor
Cleveland, Mark
Cole, James
Cole, Timothy
Costello, Walter
Cushman, Mary
Davis, Cabell
Derby, Charles
Devine, Dana
Dojiri, Masahiro
Dourdeville, Theodore
Duncan, Thomas
Eisen, Judith
Faford, Theresa
Foreman, Kenneth
French, Kathleen
Giblin, Anne
Gibson, Daniel
Greene, Sharon
Heinen, John
Hill, Russell
Howes, Brian
Jacobson, Stewart
Jordan, Thomas
Kent, Karla
Kipp, Katrina
Kolba, Clifford
Maciolek, Nancy
Ogonowski, Mark
Pascoe, Natalie
Piotrowski, Michael
Reid, Robert
Stenzler, Daniel
Thorington, Glyne
Werme, Christine
Wier, Susan
Williams, Isabelle

YEAR-IN-SCIENCE

Spring 1977

William Bowden
Colleen Cavanaugh
Hilary Morgan
James Morris
James Reed

Summer 1977

William Bowden
Coleen Cavanaugh
James Morris
James Reed

Fall 1977

William Bowden
Paul Detwiler
Michelle Dionne
Gary Gulka
James Morris
James Reed

3. Scholarships, 1977

Bio Club: Paula Marcus
Gary H. Calkins: Charlene Denys
Lucretia Crocker: Rebecca Schneider
Macy:

January

Carl B. Anderson
David Chambers
Gordon Harris
Willie J. Hawkins
Joyce Hunter
Sandra Roberts
Ronald Smith
Brenda Watkins
Donella Wilson

Summer

Yvonne Collins
Gordon Harris
Estralita Jones
Audrey Robinson
Mary G. Robinson
Paul Sandridge
Glyne Thorington
Carla Whittaker

Society of General Physiologists:

Louis Herlands
Mark Boothby
Mary Porter

4. Training Programs

Research Program in Microbial Ecology

I. Instructors

Holger W. Jannasch, Woods Hole Oceanographic Institution, director of course
Jane A. Gibson, Cornell University
Robert E. Hungate, University of California, Davis
Alex Keynan, The Hebrew University, Jerusalem
Edward R. Leadbetter, Amherst College

II. Consultants

Harlyn O. Halvorson, Brandeis University
J. Woodland Hastings, Harvard University
Roger Y. Stanier, Institut Pasteur, Paris
Edward O. Wilson, Harvard University

III. Staff Associates

Russell Cuhel, Woods Hole Oceanographic Institution
James G. Ferry, Virginia Polytechnic Institute
Helge Larsen, University of Trondheim, Norway
Jeanne S. Poindexter, Public Health Research Institute, New York
Craig D. Taylor, Woods Hole Oceanographic Institution

IV. Program Assistant

James A. Dorsch, Amherst College

V. Special Lecturers

Arnold L. Demain, Massachusetts Institute of Technology
Robert R. Finn, Cornell University
Joel C. Goldman, Woods Hole Oceanographic Institution
J. Woodland Hastings, Harvard University
Harlyn O. Halvorson, Brandeis University
Galen E. Jones, University of New Hampshire
Margot Kogut, University of London, King's College, U.K.
Kenneth H. Nealson, Scripps Institute of Oceanography
Daniel Pope, Rensselear Polytechnic Institute
John H. Ryther, Woods Hole Oceanographic Institution
Robert D. Simon, University of Rochester
Ivan Valiela, Marine Biological Laboratory
John B. Waterbury, Woods Hole Oceanographic Institution
Carl R. Woese, University of Illinois, Urbana
Meyer J. Wolin, New York Department of Public Health, Albany

VI. Trainees

*Yvonne E. Collins
David R. Dickson
*Thomas D. Durant
*Kurt W. Hanselmann
Wytold R. Lebing
Carol J. McIntyre
Dana C. Pedersen
Francis J. Sansone
*Mark A. Schneider
*Kevin R. Sowers

VII. LECTURES

H. W. JANNASCH	Introduction to microbial ecology I and II
H. W. JANNASCH	Theory and practice of the chemostat
H. W. JANNASCH	Continuous culture in microbial ecology
H. W. JANNASCH	Deep sea microbiology
H. W. JANNASCH	Topics in marine microbiology
E. R. LEADBETTER	Ways of making a living. anaerobes
E. R. LEADBETTER	Ways of making a living: aerobes
E. R. LEADBETTER	Comparative aspects of nitrate utilization
E. R. LEADBETTER	Microbiological aspects of hydrocarbon utilization
E. R. LEADBETTER	Microbial ecology and the toth surface
J. A. GIBSON	The photosynthetic bacteria I and II
J. A. GIBSON	Uptake measurements in microbial ecology
A. KEYNAN	Cell biology, a refresher I and II
R. E. HUNGATE	The rumen as an ecosystem I and II
R. E. HUNGATE	Comparative microbiology of digestion
R. E. HUNGATE	Microbial cellulose digestion
H. LARSEN	Extremely halophilic bacteria
H. LARSEN	Extreme halophilism
H. LARSEN	Microbial biochemistry of fish spoilage I and II
H. LARSEN	Anerobic respiration of trimethylamine oxide I and II
J. S. POINDEXTER	The bacterial prostheca: occurrence and ultrastructure
J. S. POINDEXTER	Obligate differentiation: *Caulobacter crescentus*
J. S. POINDEXTER	Environmental influences on how far to go
J. G. FERRY	Microbial ecology of the salt marsh
J. G. FERRY	Ecology of sulfate reduction
J. G. FERRY	Anaerobic hydrogen metabolism
C. D. TAYLOR	The biology and ecology of methane formation I and II
R. CUHEL	Psychrophilic bacteria
A. DEMAIN	Function of antibiotics in natural microbial populations
R. R. FINN	Applications of continuous culture in research
J. C. GOLDMAN	Continuous culture of algae
J. W. HASTINGS	Bacterial bioluminescence I and II
G. E. JONES	Trace metals in bacterial growth
M. KOGUT	Moderately halophilic bacteria
K. H. NEALSON	Genetics in marine bacteria
K. H. NEALSON	Autotrophy and manganese oxidizing bacteria
D. POPE	Low temperature and high pressure in bacterial growth
J. H. RYTHER	Aquaculture I and II
I. VALIELA	The Sippewisset marsh
J. B. WATERBURY	Cyanobacteria which reproduce by multiple fission
C. R. WOESE	Studies in bacterial phylogeny
M. J. WOLIN	Species interaction in anaerobic systems

5. TABULAR VIEW OF ATTENDANCE, 1973–1977

	1973	*1974*	*1975*	*1976*	*1977*
INVESTIGATORS—TOTAL	523	508	511	535	501
Independent	312	302	301	312	280
Library Readers	86	75	81	93	82
Research Assistants	125	131	129	130	139

STUDENTS—TOTAL	135	158	212	249	212
Summer courses					
Experimental Invertebrate Zoology	32	30	34	36	33
Embryology	20	21	24	24	25
Physiology	41	40	33	41	34
Experimental Marine Botany	15	11	14	16	16
Ecology	15	14	18	18	
Marine Ecology					19
Neurobiology	12	12	12	12	12
January courses					
Developmental Biology		30	20	32	14
Behavior			17	17	20
Biosphere			17		
Ecology				29	17
Neurobiology			23	24	22
TRAINEES—TOTAL	38	41	31	9	10
TOTAL ATTENDANCE	696	707	754	793	723
Less persons represented in two categories	0	0	0	1	0
	696	707	754	792	723
INSTITUTIONS REPRESENTED—TOTAL	239	222	237	234	226
FOREIGN INSTITUTIONS REPRESENTED	40	31	26	33	33

6. INSTITUTIONS REPRESENTED, 1977

Alabama, University of
Albert Einstein College of Medicine
American Museum of Natural History
Amherst College
Atlanta University
Bates College
Baylor College of Medicine
Boston College
Boston University
Boston University School of Medicine
Brandeis University
Bridgewater State College
Brooklyn College, The City University of New York
Brown University
Buena Vista College
Buxton School
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, San Francisco
California, University of, Santa Barbara
California, University of, Santa Cruz
Carnegie-Mellon University
Case Western Reserve University
Central Connecticut State College
Chatham College
Children's Hospital Medical Center, Boston
Chicago, University of
Cincinnati, University of
Cincinnati, University of, College of Medicine
City College, The City University of New York
Clark University
Cleveland State University
Colby College
Cold Spring Harbor Laboratory
College of Mount St. Joseph on the Ohio
Colorado, University of
Colorado College
Colorado Women's College
Columbia University
Connecticut, University of
Connecticut, University of, Health Center
Connecticut College
Cornell University
Dartmouth College
Delaware, University of
Delaware State College
De Paul University
De Pauw University
Dillard University
Drew University
Duke University
Duke University Medical Center
Eisenhower College
Emory University
Florida, University of
Florida A & M University
Florida State University
Framingham State College

General Electric Research and Development Center
George Washington University
Georgetown University
Goucher College
Grambling State University
Grass Foundation
Guilford College
Hahnemann Medical College
Hampshire College
Harvard Medical School
Harvard University
Hawaii, University of
Herbert Lehman College, The City University of New York
Howard University
Hunter College, The City University of New York
Illinois, University of
Illinois Institute of Technology
Indiana University
Institute for Cancer Research
Ithaca College
Jersey City State College
John B. Pierce Foundation Laboratory
Johns Hopkins University, The
Johns Hopkins University, The, School of Hygiene
Johns Hopkins University, The, School of Medicine
Kansas, University of
Kansas State University
Kentucky, University of
Kentucky State University
Kenyon College
Kresge Eye Institute
Laboratory of Sensory Physiology, MBL
Louisiana State University
Lowell, University of
Loyola College
Maine, University of, Orono
Manchester Community College
Marine Research, Inc.
Marietta College
Marine Science Institute, Nahant
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of, Amherst
Massachusetts, University of, Boston
Massachusetts, University of, Medical Center, Worcester
Massachusetts Eye and Ear Infirmary
Massachusetts General Hospital
Massachusetts Institute of Technology
Mayo Clinic
Mellon Institute of the Carnegie-Mellon University
Merck Institute for Therapeutic Research
Miami, University of
Miami, University of, School of Marine and Atmospheric Science
Miami, University of, School of Medicine
Michigan, University of
Michigan State University
Millersville State College
Minnesota, University of
Miriam Hospital, The
Montana, University of
Montefiore Hospital and Medical Center
Mount Holyoke College
National Institute of Arthritis, Metabolism and Digestive Diseases
National Institute of Mental Health
Nebraska, University of
New Hampshire, University of
New York Aquarium
New York University
New York University Medical Center
New York University School of Medicine
North Carolina, University of, at Chapel Hill
North Carolina State University, Raleigh
North Dakota, University of
Northeastern University
Northwestern University
Notre Dame, University of
Oakland University
Oberlin College
Ohio State University
Oregon, University of
Oregon State University
Osborn Laboratories of Marine Sciences
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pennsylvania, University of, School of Veterinary Medicine
Pennsylvania State University
Pittsburgh, University of
Pittsburgh, University of, School of Medicine
Population Council, The
Princeton University
Purdue University
Radcliffe College
Reed College
Rhode Island, University of
Rice University
Rochester, University of
Rochester, University of, School of Medicine and Dentistry
Rockefeller University, The
Rollins College
Rush Medical College
Russell Sage College
Rutgers—The State University of New Jersey
Rutgers University Medical School
St. Jude Children's Research Hospital
St. Lawrence University

Seton Hill College
Shriners Burns Institute
Sidney Farber Cancer Center
Siena College
Simmons College
Smith College
South Florida, University of
Southampton College
Stanford University
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Binghamton
State University of New York at Buffalo
State University of New York at Stony Brook
State University of New York at Syracuse
Suffolk University
Swarthmore College
Syracuse University
Temple University
Temple University Medical School
Tennessee, University of
Tennessee State University
Texas Southern University
Texas, University of, Austin
Texas, University of, Medical Branch
Thomas Jefferson University
Trinity College
Tufts University
Tulane University
U. S. Army Environmental Hygiene Agency
Upsala College
Upjohn Company, The
Utah, University of
Vanderbilt University
Vanderbilt University School of Medicine
Vermont, University of
Veteran's Administration Hospital, Brooklyn
Virginia, University of
Virginia, University of, School of Medicine
Virginia Polytechnic Institute
Washington & Jefferson College
Washington, University of
Washington University, St. Louis
Washington University, School of Medicine
Wayne State University
Wesleyan University
Wheaton College
Wisconsin, University of
Wistar Institute
Woods Hole Oceanographic Institution
Worcester Foundation Experimental Biology
Yale University
Yale University School of Medicine
Yeshiva University

Foreign Institutions Represented, 1977

Aarhus, University of, Denmark
Basel, The University of, Switzerland
Calgary, University of, Canada
Cambridge University, England
Caribbean Marine Biological Institute, Netherlands
Colegio Universitario de Humacai, Puerto Rico
College de France, France
Dalhousie University, Canada
Edinburgh, University of, Scotland
Flinders University of South Australia, Australia
Glasgow, University of, Scotland
Guelph, University of, Canada
Hebrew University, The, Israel
Institut fur Neurobiologie der KFA, West Germany
Instituto Venezolano de Investigaciones, Venezuela
King's College, London, England
Leeds, University of, England
London, University of, England
Lund, University of, Sweden
McGill University, Canada
National University of Mexico, Mexico
North Wales, University of, Wales
Ottawa, University of, Canada
Philips Research Laboratory, Holland
Queen Mary College, London, England
Rijksuniversiteit Groningen, The Netherlands
Scarborough College, Canada
Toronto, University of, Canada
Trondheim, University of, Norway
Universidad Nacional Autonoma de Mexico, Mexico
Universita Cattolica Facolta Dimedicina, Italy
Université Laval, Canada
University College, London, England
Zurich, University of, Switzerland

7. Friday Evening Lectures, 1977

June 24

Helge Larsen.................. Gas vacuoles in bacteria
Norwegian Institute of
Technology; University of
Trondheim; Rand Fellow, MBL

July 1

Perry W. Gilbert..............Sharks as barbarians and benefactors
Cornell University;
Mote Marine Laboratory

July 8

Daniel Tosteson...............Lithium, membranes and mania
Harvard Medical School

July 14

Philippe Ascher................Acetylcholine-induced channels in *Aplysia* neurons
Laboratoire de Neurobiologie,
Ecole Normale Supérieure, Paris;
Alexander Forbes Lecturer, MBL

July 15

JacSue Kehoe..................Hidden receptors in *Aplysia* neurons
Laboratoire de Neurobiologie,
Ecole Normale Supérieure, Paris;
Alexander Forbes Lecturer, MBL

July 22

Lincoln P. Brower.............The ecological chemistry of the monarch butterfly
Amherst College

July 29

Bert W. O'Malley.............Studies on the regulation of gene expression in eucaryotes
Baylor College of Medicine

August 5

Philip Leder...................Cloning specific segments of the mammalian genome: bacteriophage lambda containing mouse globin and surrounding gene sequences
National Institute of Child
Health and Human Development

August 12

Leon D. Harmon...............Artificial intelligence and natural stupidity
Case Western Reserve University

August 19

Clara Franzini-Armstrong.....Muscle membranes and excitation-contraction coupling
University of Pennsylvania

August 26

Howard L. Sanders............Evolutionary ecology and the deep-sea benthos
Woods Hole Oceanographic
Institution

8. MEMBERS OF THE CORPORATION, 1977

Including Action of 1977 Annual Meeting

Life Members

ADOLPH, DR. EDWARD F., University of Rochester, School of Medicine and Dentistry, Rochester, New York 14627

BARTH, DR. LESTER G., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

BEAM, DR. HAROLD W., Department of Zoology, State University of Iowa, Iowa City, Iowa 52240

BEHRE, DR. ELLINOR H., Black Mountain, North Carolina 28711

BERTHOLF, DR. LLOYD M., 307 Phoenix Ave., Bloomington, Illinois 61701

BODANSKY, DR. OSCAR, 16 Hawks Nest Road, Stony Brook, New York 11790

BOLD, DR. HAROLD C., Department of Botany, University of Texas, Austin, Texas 78712

BRIDGMAN, DR. A. JOSEPHINE, 715 Kirk Rd., Decatur, Georgia 30030

BROWN, DR. DUGALD E. S., Cape Haze, Box 426, Placida, Florida 33946

BURDICK, DR. C. LALOR, The Lalor Foundation, 4400 Lancaster Pike, Wilmington, Delaware 19805

CARPENTER, DR. RUSSELL L., 60 Lake St., Winchester, Massachusetss 01890

CLARKE, DR. GEORGE L., 44 Juniper Road, Belmont, Massachusetts 02178

CLEMENT, DR. ANTHONY C., Department of Biology, Emory University, Atlanta, Georgia 30322

COLWIN, DR. ARTHUR L., 320 Woodcrest Rd., Key Biscayne, Florida 33149

COLWIN, DR. LAURA H., 320 Woodcrest Rd., Key Biscayne, Florida 33149

COSTELLO, DR. HELEN M., Morgan Rd., Woods Hole, Massachusetts 02543

CROUSE, DR. HELEN V., Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

DILLER, DR. IRENE C., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038

DILLER, DR. WILLIAM F., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038

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VI. REPORT OF THE LIBRARIAN

In 1977 the library applied to the Regional Medical Library in Boston to become a MEDLARS Search Center for the medical community of Cape Cod and the Islands. MEDLARS is a computer based retrieval system of the National Library of Medicine, which provides immediate access to over half a million citations for the biomedical journals. Our library holds 760 medical journals in a collection of over 5,000 journal titles, and we are now offering this on-line access to the literature to the members of the health services on the Cape and Islands. A staff member, Judy Ashmore, attended two training sessions in Washington, and we offered the service to our own scientists during the summer months. We have demonstrated the system at the Falmouth, Hyannis, Martha's Vineyard and Tobey Hospitals and also at two Media Sessions at the Health Resources Center at the Cape Cod Community College. The physicians and nurses in the area seem very pleased with this new service and have used it extensively. In addition to the medical data bases, we also offer access to the citations contained in the Lockheed and Bibliographic Retrieval Services data banks. Three members of the library staff are now trained to offer literature searches.

Space is now a critical problem. Room 304 was taken from the library for administrative office space, and we were not granted Room 205 (next to the library office), a Library Committee request of long standing. Many journals are now shelved out of alphabetical order, and work space for the staff services is very crowded. Both MBL and WHOI are adding hundreds of volumes every year, and the collection is expanding rapidly. We added 98 new journal titles this year, 50 of them through exchange arrangements. The total number of volumes held in the library is now over 164,000.

VII. REPORT OF THE TREASURER

The Marine Biological Laboratory reported an operating loss of $85,414 in 1977, exclusive of a depreciation charge of $281,291, as compared to a surplus of $106,197 in 1976. As you recall from the Treasurer's report last year, approximately $110,000 of revenues recognized in 1976 was applicable to services rendered in 1975. Therefore, the loss for 1977 is comparable to approximately break-even operations in 1976.

Total revenues in 1977 amounted to $2,081,494, an increase of $161,000 over the prior year. Increases in tuition, dormitory and dining hall revenues were approximately offset by a decline in laboratory rentals due to recording the retroactive adjustment in 1976 revenues.

Investment income in 1977 amounted to $321,915, an increase of 6.5% over 1976. At December 31, 1977, the Laboratory's restricted and unrestricted investments amounted to $5,396,175 at cost and $5,379,045 at market value. The Budget Committee has recently commenced an evaluation of various alternatives aimed at maximizing investment income and increasing the total return from endowment funds.

Unrestricted gifts in 1977 amounted to $158,850, an increase of 65% over 1976. The Laboratory was the beneficiary of substantial grants from four foundations, which represents a new record level of giving. All of us associated with the Laboratory are most grateful for this very generous financial support.

Operating expenditures increased 19% to $2,166,908. However, a substantial portion of the increase was associated with new courses that generated revenue that more than offset the incremental expenses. However, like all organizations, the Laboratory's general expense for salaries, utilities and other ongoing costs increased at approximately the same rate as the increase in the cost of living.

During the past several months, the Executive Committee has made an intensive analysis of the Laboratory's revenue sources and operating expenditures. The conclusion of its analysis is that the Laboratory is operating at peak capacity during its summer months and tuition and laboratory rentals cannot be further increased without substantially impairing the utilization of facilities. Operating expenditures have been carefully reviewed and budgeted recurring operating expenditures for 1978 are only 5% greater than actual expenditures for 1977.

During 1978, the Laboratory will develop a more detailed and comprehensive program to increase the amount of unrestricted gifts and will introduce additional programs for utilizing laboratory space and dormitories during the non-summer months. We are confident that these efforts will result in improved financial results for the Laboratory in 1978.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of Marine Biological Laboratory as of December 31, 1977 and 1976, and the related statements of current funds revenues, expenditures, and other changes and changes in fund balances for the years then ended. Our examinations were made in accordance with generally accepted auditing standards and, accordingly, included confirmation from the custodians of securities owned at December 31, 1977 and 1976, and such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

As more fully described in Note B to the financial statements, the Laboratory excludes certain costs of buildings and equipment from the balance sheet. In our opinion, generally accepted accounting principles require that such costs be included as investment in plant in the financial statements.

In our opinion, except for the effects on the financial statements of the matter discussed in the preceding paragraph, the aforementioned financial statements present fairly the financial position of Marine Biological Laboratory at December 31, 1977 and 1976, and its current funds revenues, expenditures and other changes and the changes in fund balances for the years then ended, in conformity with generally accepted accounting principles applied on a consistent basis.

The summary of investments included herein was obtained from the Laboratory's records in the course of our examination and, in our opinion, is fairly stated in all material respects in relation to the basic financial statements taken as a whole.

Boston, Massachusetts
April 7, 1978

COOPERS & LYBRAND

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1977 and 1976

Assets	*1977*	*1976*
Current Funds:		
Unrestricted:		
Cash, including deposits at interest	$ 517,513	$ 213,950
Accounts receivable, net of allowance for uncollectible accounts of $12,955 in 1977 and $15,566 in 1976	540,743	446,741
Other assets	15,996	6,141
Due to restricted current funds	(294,485)	(124,371)
Due from invested funds	105,133	65,557
Total unrestricted	884,900	608,018
Restricted:		
Cash	87,013	1,959
Investments, at cost; market value: 1977—$1,118,917; 1976—$1,339,892 (Note A, Schedule I)	1,112,683	1,305,084
Due from unrestricted current fund	294,485	124,371
Due from invested funds	173,191	136,124
Total restricted	1,667,372	1,567,538
Total current funds	$ 2,552,272	$ 2,175,556
Invested Funds:		
Cash	102,031	3,297
Accounts receivable	—	1,435
Investments, at cost; market value: 1977—$3,799,434; 1976—$4,482,948 (Note A, Schedule I)	3,822,798	4,092,770
Due to unrestricted current fund	(105,133)	(65,557)
Due to restricted current funds	(173,191)	(136,124)
Total invested funds	$ 3,646,505	$ 3,895,821
Plant Fund:		
Land, buildings and equipment at cost (Note B)	12,374,587	12,345,587
Less accumulated depreciation	3,778,545	3,497,254
Total plant fund	$ 8,596,042	$ 8,848,333

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Balance Sheet

December 31, 1977 and 1976

Liabilities and Fund Balances	*1977*	*1976*
Current Funds:		
Unrestricted:		
Accounts payable and accrued expenses	$ 195,418	$ 173,387
Deferred income	62,131	60,335
Fund balance	627,351	374,296
Total unrestricted	884,900	608,018
Restricted:		
Fund balances:		
Unexpended gifts and grants	1,597,841	1,507,577
Unexpended income of endowment funds	69,531	59,961
Total restricted	1,667,372	1,567,538
Total current funds	$ 2,552,272	$ 2,175,556
Invested Funds:		
Endowment funds	2,172,160	2,147,067
Quasi-endowment funds	934,143	1,260,509
Retirement fund (Note C)	540,202	488,245
Total invested funds	$ 3,646,505	$ 3,895,821
Plant Fund:		
Invested in plant	8,596,042	8,848,333
Total plant fund	$ 8,596,042	$ 8,848,333

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CURRENT FUNDS REVENUES, EXPENDITURES, AND OTHER CHANGES

for the years ended December 31, 1977 and 1976

	Unrestricted		*Restricted*		*Total*	
	1977	*1976*	*1977*	*1976*	*1977*	*1976*
Revenues:						
Instruction:						
Tuition	$ 164,495	$ 129,605	$ 35,200	$ 43,100	$ 199,695	$ 172,705
Grants and contracts:						
Government	7,468	5,394	128,197	125,301	135,665	130,695
Private			33,258	26,916	33,258	26,916
Research (Note D):						
Laboratory rentals	424,631	483,952			424,631	483,952
Grants and contracts:						
Government	166,970	192,101	646,497	373,454	813,467	565,555
Private	84,917	132,115	457,896	473,181	542,813	605,296
Dormitory	285,285	245,349			285,285	245,349
Dining Hall	134,454	38,985			134,454	38,985
Library	126,652	120,318			126,652	120,318
Biological Bulletin	85,430	71,727			85,430	71,727
Support departments:						
Research services	142,420	138,771			142,420	138,771
Supply	85,131	80,104			85,131	80,104
Investment income	158,361	147,625	39,359	28,016	197,720	175,641
Gifts	158,850	96,185	101,013	105,834	259,863	202,019
Other	56,430	38,291			56,430	38,291
Total revenues	2,081,494	1,920,522	1,441,420	1,175,802	3,522,914	3,096,324

	Unrestricted		*Restricted*		*Total*	
	1977	*1976*	*1977*	*1976*	*1977*	*1976*
Operating expenditures:						
Instruction	123,037	86,351	71,349	109,347	194,386	195,698
Research	1,386	5,029	1,104,393	846,635	1,105,779	851,664
Scholarships and stipends			216,736	158,982	216,736	158,982
Dormitory	140,358	111,590			140,358	111,590
Dining Hall	130,349	36,300			130,349	36,300
Library	208,149	185,669	22,043	19,075	230,192	204,744
Biological Bulletin	77,165	79,625			77,165	79,625
Support departments:						
Research services	305,847	243,891	20,415	2,500	326,262	246,391
Supply	216,278	197,919	255	45	216,533	197,964
Administration	462,479	398,868	4,048	4,671	466,527	403,539
Plant operation	501,860	469,083	2,181	34,547	504,041	503,630
Total expenditures	2,166,908	1,814,325	1,441,420	1,175,802	3,608,328	2,990,127
Excess of (expenditures) revenues	(85,414)	106,197	—	—	(85,414)	106,197
Transfers and additions:						
Excess of restricted gifts, grants and investment income received over amounts expended			99,834	128,337	99,834	128,337
From plant fund—proceeds of sale of plant assets	3,000	29,040			3,000	29,040
From quasi-endowment fund	335,469	199,800			335,469	199,800
Net transfers and additions	338,469	228,840	99,834	128,337	438,303	357,177
Net increase in fund balances	$ 253,055	$ 335,037	$ 99,834	$ 128,337	$ 352,889	$ 463,374

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Statements of Changes in Fund Balances

for the years ended December 31, 1977 and 1976

	Current Funds		*Invested Funds*			
1976	*Unrestricted*	*Restricted*	*Endowment*	*Quasi-Endowment*	*Retirement*	*Plant Fund*
Balances at December 31, 1975	$ 39,259	$1,439,201	$1,905,920	$1,462,894	$ 438,255	$9,129,270
Increases:						
Unrestricted current fund revenues	1,920,522					
Grants and gifts		1,453,746	62,830			
Realized net gains (losses) on sale of investments			178,317	(2,585)		
Investment income		136,903			17,794	
Proceeds of sale of equipment						29,040
Addition to pension fund					52,136	
Tuition		43,100				
Decreases:						
Instruction, research and general expenditures	(1,814,325)	(1,175,802)				
Book value of equipment sold						(386)
Indirect costs		(329,610)				
Payments to pensioners					(19,940)	
Depreciation						(280,551)
Transfers—additions (deductions):						
Proceeds of sale of equipment	29,040					(29,040)
Transfer to current funds	199,800			(199,800)		

1977	Current Funds		Invested Funds			
	Unrestricted	*Restricted*	*Endowment*	*Quasi-Endowment*	*Retirement*	*Plant Fund*
Balances at December 31, 1976	374,296	1,567,538	2,147,067	1,260,509	488,245	8,848,333
Increases:						
Unrestricted current fund revenues	2,081,494					
Grants and gifts		1,622,821				29,000
Realized net gains on sale of investments			25,093	9,103		
Investment income		142,588			20,966	
Proceeds of sale of equipment						3,000
Addition to pension fund					50,150	
Tuition		35,200				
Decreases:						
Instruction, research and general expenditures	(2,166,908)	(1,441,420)				
Indirect costs		(259,355)				
Payments to pensioners					(19,159)	
Depreciation						(281,291)
Transfers—additions (deductions):						
Proceeds of sale of equipment	3,000					(3,000)
Transfer to current funds	335,469			(335,469)		
Balances at December 31, 1977	$ 627,351	$1,667,372	$2,172,160	$ 934,143	$ 540,202	$8,596,042

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Notes to Financial Statements

A. *Significant Accounting Policies:*

Basis of Presentation—Fund Accounting

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with activities or objectives specified. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted funds may only be utilized in accordance with the purposes established by the source of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating purposes. Unrestricted revenue is reported as revenue in the unrestricted current fund when received.

Endowment funds are subject to restrictions requiring that the principal be invested and only the income utilized. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds, however, any portion of these funds may be expended.

Investments

Investments purchased by the Laboratory are carried at cost. Investments donated to the Laboratory are carried at fair market value at date received. For determination of gain or loss upon disposal, cost is determined based on the specific identification method.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of the market value at the beginning of the quarter, adjusted for the cost of any additions or disposals during the quarter.

Indirect Cost Recovery

Through 1976, the Laboratory recovered indirect costs by charging occupants of laboratory space fees based on an estimated indirect cost rate for the period the space was occupied. The estimated rate was adjusted on a retroactive basis upon determination of actual indirect costs for the year. Beginning in 1977, fixed rates are used currently. When actual rates are subsequently determined, the difference is reflected in the next fixed rate negotiated.

B. *Land, Buildings and Equipment:*

Following is a summary of the plant fund assets:

Classification	*1977*	*1976*
Land	$ 639,693	$ 639,693
Buildings	10,143,088	10,143,088
Equipment	1,591,806	1,562,806
	12,374,587	12,345,587
Less accumulated depreciation	3,778,545	3,497,254
	$ 8,596,042	$ 8,848,333

The original cost of land, buildings and related initial furnishing equipment is capitalized when the assets are acquired. The cost of subsequent additions and purchases, repairs and remodeling is expensed when incurred. Equipment and remodeling expenditures amounted to approximately $110,000 and $52,000 in 1977 and 1976, respectively.

Depreciation is computed using the straight-line method over estimated useful lives of 40 years for buildings and 20 years for equipment.

C. *Retirement Fund:*

The Laboratory has a noncontributory pension plan for substantially all full-time employees which complies with the requirements of the Employee Retirement Income Security Act of 1974. The actuarially determined pension expenses charged to operations in 1977 and 1976 were $64,277 and $54,973, respectively. The Laboratory's policy is to fund pension costs accrued.

D. *Research Revenues:*

The 1976 research revenues include approximately $111,000 of retroactive overhead rate adjustments to amounts previously billed in 1975, as described in Note A.

E. *Pledges and Grants:*

As of December 31, 1977 and 1976, the following amounts remain to be received from previous gifts and grants for specific research and instruction programs, and are expected to be received as follows:

	December 31	
	1977	*1976*
1977		$ 891,000
1978	$1,697,486	269,000
1979	115,000	114,000
1980		6,000
	$1,812,486	$1,280,000

SCHEDULE I

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1977 and 1976

	Cost		*Market*		*Investment Income*	
	1977	*1976*	*1977*	*1976*	*1977*	*1976*
Invested Funds:						
U. S. Government securities	$ 444,638	$ 603,324	$ 448,295	$ 626,116	$ 40,923	$ 17,714
Corporate bonds	770,012	914,634	676,789	801,032	48,681	52,191
Common stocks	2,080,655	2,112,140	2,189,492	2,597,619	125,462	112,465
Commercial paper	—	326,367	—	326,367 (1)	10,990	21,823
Preferred stocks	83,032	118,756	61,072	114,265	4,468	5,229
Units in combined pension fund	426,912	—	406,237	—	9,394	—
Real estate	17,549	17,549	17,549 (1)	17,549 (1)		
Total	$3,822,798	$4,092,770	$3,799,434	$4,482,948	239,918	209,422
Less custodian fees					11,835	10,760
					228,083	198,662
Current Restricted Funds:						
U. S. Government securities	1,112,683	1,297,254	1,118,917	1,332,062		
Certificate of deposit	—	7,830	—	7,830 (1)		
Total	$1,112,683	$1,305,084	$1,118,917	$1,339,892	86,748	99,126

Current Unrestricted Funds:		
Earned on corporate savings account	7,084	4,534
Net investment income	$321,915	$302,322
Disposition of investment income:		
Restricted for current use:		
Research	95,789	101,985
Scholarships	25,289	17,067
Library	21,510	17,851
	142,588 (2)	136,903 (2)
Retirement fund	20,966	17,794
Unrestricted—utilized in current operations	158,361	147,625
	$321,915	$302,322

(1) At cost.

(2) $39,359 in 1977 and $28,016 in 1976 was utilized in current restricted operations.

Reference: *Biol. Bull.*, **155**: 98–112. (August, 1978)

AGRAULIS AND *PASSIFLORA* I. CONTROL OF SPECIFICITY[1]

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Butterflies in the tribe Heliconiini (Nymphalidae) exhibit a highly specific association with one family of dicotyledonous plants, the Passifloraceae (passion-vines). Research on heliconians and passion-vines at the community level suggests that many aspects of the butterflies' sophisticated behavioral capabilities and sensory systems have evolved in the context of the exploitation of larval food-plant resources, and that the passion-vines have responded in turn with a diverse array of morphological "defenses" against heliconian predation (Benson, Brown, and Gilbert, 1975; Gilbert, 1975). This constitutes an extension of Ehrlich and Raven's (1964) proposed model for the coevolution of butterfly herbivores and their plant hosts, which considered only the stepwise proliferation by the plant and subsequent circumvention by an insect of chemical barriers to herbivory.

In spite of extensive observations on the natural history and general behavior of heliconians (Crane, 1955, 1957; Alexander, 1961a, b), information concerning the sensory modalities and behavior involved in the recogntion of specific host-plants by heliconians remains to a great extent anecdotal. No experimental investigation into the methods of host-plant selection by a single species of heliconian has been undertaken to our knowledge. Such information is critical in testing postulated cause-effect relationships between heliconian behavior and *Passiflora* morphology.

This problem was approached in regard to the heliconian *Agraulis vanillae* and its passion-vine host plants. In the neotropics, *Agraulis* preferentially infests vines in the *Passiflora* subgenus *Granadilla* (Benson et al., 1975).

The following experiments examine the role of butterfly behavior and host-specific mortality in determining the infestation levels of various species of *Passiflora* by *Agraulis* in Santa Barbara, California.

MATERIALS AND METHODS

The butterflies

Laboratory cultures of *Agraulis vanillae incarnata* (Riley), started in the spring of 1973 from wild stock collected locally in Santa Barbara, California, were maintained in a controlled-environment room on a 15L:9D cycle at 23° C and 60% humidity. Adults were placed in nylon screened cages 61 cm square and 91 cm high. In these cages they fed either on 1 M sucrose in sponges or on a 20% honey solution in artificial red flowers which was changed every other

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[1] This work was supported by National Science Foundation Grant GB 41210.

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day. A small piece of red tape in the center of the sponges increased their attractiveness to the adults.

In addition to the overhead Cool-White fluorescent room lights, each cage was illuminated by one 150W–120V GE reflector flood, suspended 25 cm above the top of the cage. These lights cycled on a 13L : 11D regime coordinated with the room lights to provide one hour of "dawn" and one hour of "twilight." Under these conditions the average adult life span was 10–16 days. The adults adjusted well to caged conditions. Apparently, mating and oviposition proceeded normally. Females oviposited on fresh new-growth shoots of *Passiflora*. Eggs on these shoots were then transferred to wire sceen cylinders (19.5 cm in diameter, 45 cm high) closed at the top and bottom by the cover and bowl of a plastic cake pan. Fresh vine of the species on which the larvae had hatched was provided as necessary until pupation. Shortly after emergence the adults were transferrd from these wire cylinders to the sceen cages.

Females laid very few eggs on two species of *Passiflora: P. mollissima* and *P. edulis*. Larval cultures on these two species were established by transferring newly hatched larvae from the screen of the adult cages (where females frequently oviposited) to fresh new-growth shoots of these vines.

Once a year, in the summer, butterflies from the local wild population were added to the laboratory cultures to maintain a high frequency of wild-type genes.

The plants

Six species of *Passiflora*, each locally available in Santa Barbara County, were used in the investigation: *P. alato-caerulea*, hereinafter referred to as AC, (the familiar garden hybrid, *P. alata* × *P. caerulea*, both parental types in the subgenus *Granadilla*); *P. caerulea* (referred to as CR); *P. manicata* (referred to as MN; subgenus *Granadillastrum*); *P. mollissima* (referred to as ML; subgenus *Tacsonia*); *P. edulis* (referred to as ED; subgenus *Granadilla*); and a hybrid of unknown origin, *Passiflora* sp. (referred to as SP), which is morphologically intermediate between *P. manicata* and *P. mollissima* (Killip, 1938). None of these is native to California, atlhough *P. manicata* may occur as a garden escape. All specimens were located in the gardens of private residences. For any one species the same specimen of vine provided material for all experiments and cultures. Occasionally alternate sources for a vine species replaced the standard source if it became unavailable.

The species of vine which serves as the larval food plant for an experimental sample of butterflies is hereinafter referred to as the insects' "host" species. Butterfly lineages on the different species of *Passiflora* were not kept pure except where specified. Eggs used to establish one culture were generally contributed by adults from several other cultures. This mixing of lineages helped to prevent the formation of separate strains of butterflies each adapted to a different host-plant species.

Larval food choice experiments

The larvae of many temperate zone lepidopterans exhibit feeding preferences which vary according to feeding experience in earlier instars (Jermy, Hanson, and Dethier, 1968; Wiklund, 1973; Yamamoto, 1974; Städler and Hanson, University of Maryland, personal communication). Such preferences may affect host-plant selection either directly as a result of the larvae moving from one species of plant to another (Hanson, University of Maryland, personal communication) or indirectly by affecting the ovipositional preferences of subsequent adult females as stated in Hopkins' host selection principle (Hopkins, 1917; Craighead, 1921). The induction of feeding preferences in the larvae of *Agraulis* was tested for in the following manner.

Fourth-instar larvae, raised exclusively on one species of *Passiflora*, were tested in single choice situations matching agar plates containing the mashed leaves of one species of *Passiflora* against agar plates containing mashed leaves of another species. This technique necessarily eliminates any influence of nonchemical factors such as leaf surface texture on feeding specificity; however, all experiments known to us indicate that chemical agents primarily determine the specificity of feeding responses of larval lepidopterans and most other oligophagous insects (*e.g.,* Dethier, 1966; Schoonhoven, 1968).

The plates containing ground leaves of *Passiflora* were prepared as follows: 7 grams of Bactoagar were dissolved in 210 ml boiling water and the solution cooled to 68° C. Eight grams of fresh leaves, taken from new-growth shoots about 18 inches long, were ground in 100 ml water in a blender for 30 seconds. The cooled agar solution was added to this unfiltered leaf mash and blended for an additional 15 seconds. "Unlabeled" agar plates contained exactly the same formula without leaf mash.

The warm agar-leaf mixture was poured in a 3 mm thick layer into two diagonally opposed quadrants of a 25 cm diameter glass finger bowl. The remaining two quadrants received either an unlabeled agar solution or one containing leaf mash of a different species of *Passiflora.* The agar surfaces in adjacent quadrants abutted against each other and, when all were labeled with leaf mash, showed little discernable difference in color or texture. A plastic ring 7 mm high was inserted in a 5 cm diameter circle cleared of agar in the center of the bowl where all four quadrants met. After being placed in this ring larvae tended to mount it and walk some distance around it before moving onto the agar, thus increasing the probability of larval contact with both types of label before feeding.

A single experimental trial consisted of placing five fourth-instar larvae in the ring of an experimental dish and counting the number of fecal pellets deposited within each quadrant after 24 hours of feeding. The larvae had been removed from their host-plant four hours prior to an experiment, during which time they voided residual frass. This ensured that a count of the fecal pellets left in each quadrant of the experimental dishes at the end of 24 hours gave a quantitative measure of the feeding that had taken place on that quadrant during the experiment. Ten experimental trials (*i.e.,* fifty larvae) comprised each choice situation.

Food choice experiments for a single culture consisted of pairing the host-plant species of that culture with each of the other five species. All experiments were conducted in the culture laboratory (23° C, 60% humidity). Control tests, in which a total of fifty larvae (i.e. ten experimental trials) faced a feeding choice between quadrants labeled with mashed leaves of CR and unlabelled quadrants, were carried out either in the dark or with green paper taped to the underside of the bowls to control for visual cues.

Adult ovipositional choice experiments

Female specimens of *Agraulis*, raised as larvae on only one species of *Passiflora*, oviposited in single choice experiments which matched the female's larval food plant with one of the other five species. Choice situations consisted of placing four new-growth shoots of *Passiflora*, two from the host species and two from one of the other five, in 50 ml flasks of water and arranging them alternately in the back of a cage. Adjusting the number of leaves on each 18-inch sprig controlled for differences in leaf-surface area. The sprigs remained in place for 24 hours, were then reversed in order and left an additional 24 hours. The number of eggs on each species of vine was then counted. Since the females fly for a time after the deposition of each egg, significant differences in total egg count between two species of vine may be interpreted as an ovipositional preference.

Initially, only ten females, each three- to six-days old, comprised each experimental trial. But it soon became apparent that neither the number of females nor their age affected ovipositional choice behavior. Each test thereafter involved a minimum of ten females, each at least three days old. A total of fifty females was tested in at least three replicates of each choice situation. No female encountered the same choice more than once. The butterflies were allowed to feed on 1 M sucrose throughout the experiment. Males were not excluded.

Suitability of Passiflora *as a food plant*

In addition to larval feeding or adult ovipositional preferences, a potentially significant factor affecting the infestation levels of various species of *Passiflora* is the suitability of a particular plant species in terms of the insect's growth and development. In the most extreme cases adult females of several species of insect (including two heliconians—Alexander, 1961b; Benson *et al.*, 1975) have been observed to oviposit on plants toxic to the larvae or on which the larvae will not feed (Straatman, 1962, Sevastopuló, 1964). But toxins, feeding inhibitors, or nutritional imbalances need not be lethal to affect the host-plant range of an insect species. Sublethal toxins and nutritional deficiencies may reduce insect infestation of a particular plant by increasing larval mortality, lengthening larval life, increasing the number of dwarfed adults (which, in butterflies, may reduce mating success), and decreasing fecundity.

These parameters were measured in specimens of *Agraulis* raised on the various local species of *Passiflora* to determine their influence on observed patterns of host-plant infestation.

Larvae, previously unexposed to passion-vine, were collected as they hatched from eggs laid on the nylon screen of the cages by females that had been raised as larvae on either CR, or SP or, in the second experiment, on MN. Thirty first-instar larvae were placed in each of six 5-gallon plastic buckets which contained fresh new-growth shoots of one of the six species of *Passiflora*. Each container was covered with mosquito netting which prevented the larvae from escaping. The food plant material stood in 50 ml flasks of water and was replaced with fresh vine every other day to avoid excessive deterioration of the plant or larval food shortages. The same specimen of vine provided all leaf material for any one culture throughout all phases of the experiment. As larvae reached the third instar each culture was divided equally between two containers to alleviate overcrowding.

The larvae pupated in their containers. On emergence the adults were transferred to the nylon-screen cages, fed 1 M sucrose from sponges, and the females were allowed to oviposit on fresh new-growth sprigs of AC vine (the species most preferred for oviposition). These cultures were maintained in the laboratory under the conditions outlined previously.

The following parameters were measured; larval and pupal mortality, length of larval life (time, in days, from hatching to 50% pupation), rate of frass production (frass collected, dried, and weighed every other day), adult size (longest dimension of a forewing), and the number of eggs laid by the females. Each of these parameters has previously been used to indicate the suitability of a phytophagous insect's diet (Dethier, 1954; Waldbauer, 1962; Latheef and Harcourt, 1972; Wiklund, 1973; Erickson and Feeney, 1974).

Field survey of Passiflora *infestation*

A survey of passion-vines in the field was undertaken to evaluate the relative contribution of larval feeding preferences, adult ovipositional preferences, and host-specific patterns of mortality to the distribution of *Agraulis* over various species of *Passiflora*.

The survey took place during the summer of 1974 and involved 45 passion-vines (representing 6 species) located in the cities of Goleta and Santa Barbara, California. Each vine was periodically surveyed by estimating its volume and counting all the specimens of the various life history stages of *Agraulis* found on it. Except for a few vines sampled in May, sampling proceeded once every three weeks from June through August. Thus, each specimen of vine received three to four visits during the peak flight season.

RESULTS

Larval food choice experiments

In the control experiments, fourth-instar larvae consistently deposited more fecal pellets on the agar quadrants labeled with leaves of CR than on the unlabeled quadrants (totals for all ten trials of 454 and 139, respectively; $P < 0.001$; Student's *t*-test). These data correlated well with subjective evaluations of the amount of feeding in each pair of quadrants. Since the larvae voided residual frass prior to the experiment, a significant difference in the number of fecal pellets on quadrants with different labels is interpreted as a feeding choice. The number of pellets on the unlabeled agar is interpreted to be primarily the result of deposition by larvae that wandered after feeding.

Results of the food choice experiments for larvae from each of the five cultures

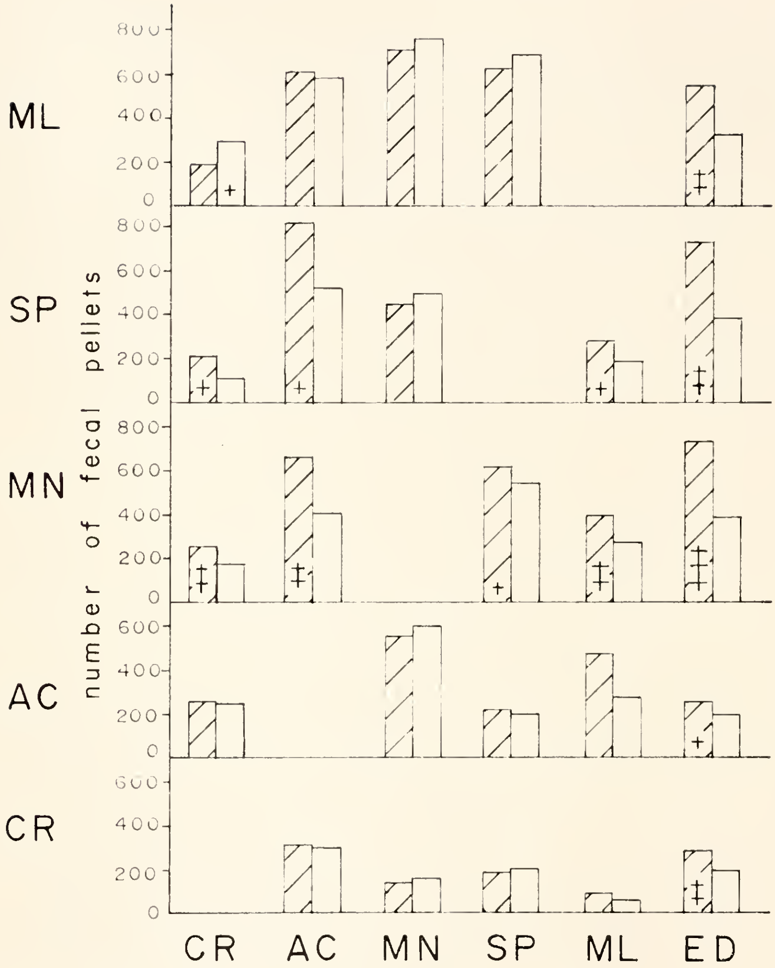

FIGURE 1. Results of larval food choice experiments. Shaded bars indicate number of fecal pellets on agar labeled with host-plant; and open bars, the number of fecal pellets on agar labeled with test plants. Host-plant species appear on the ordinate and test plants on the abscissa. The crosses indicate a significant difference between members of a pair of bars calculated by Student's *t*-test for paired comparisons: +, $0.05 > P > 0.01$; ++, $0.01 > P > 0.001$; +++, $P > 0.001$. Data are not available for ED since larvae did not survive to the fourth instar on that species. CR represents *Passiflora caerulea;* AC, *P. alato-caerulea;* MN, *P. manicata;* SP, *P.* sp., ML, *P. mollissima;* and ED, *P. edulis.*

are presented in Figure 1. Data for an ED culture are not available, since feeding was minimal and most larvae failed to survive to the fourth-instar on that species of vine.

Comparing, in Figure 1, the choice situations MN *vs.* AC and MN *vs.* CR with CR *vs.* MN and AC *vs.* MN indicates that larvae raised on MN became induced to prefer that species by the fourth-instar. Similarly, larvae reared on

SP became induced to prefer SP over AC and CR. Larvae raised either on AC or CR showed no such induced preferences. Their choices against ED may indicate an avoidance of ED rather than an induced preference for their respective hosts. It should be noted that all larvae chose their host-plant over ED.

These results suggest that MN and SP differ chemically from CR and AC, such that only larvae raised on MN or SP become induced to prefer their host-plants. Two hypotheses explaining this phenomenon are: first, leaves of MN and SP vines contain some compound(s) which effects induction of host preferences in larvae fed these species; and secondly, leaves of CR and AC vine contain an inhibitory factor to which larvae reared on CR or AC become habituated, but to which MN- and SP-raised larvae remain sensitive. The results of preliminary experiments designed to test these hypotheses were inconclusive but tended to support an intermediate interpretation: that CR leaves contained a feeding inhibitor to

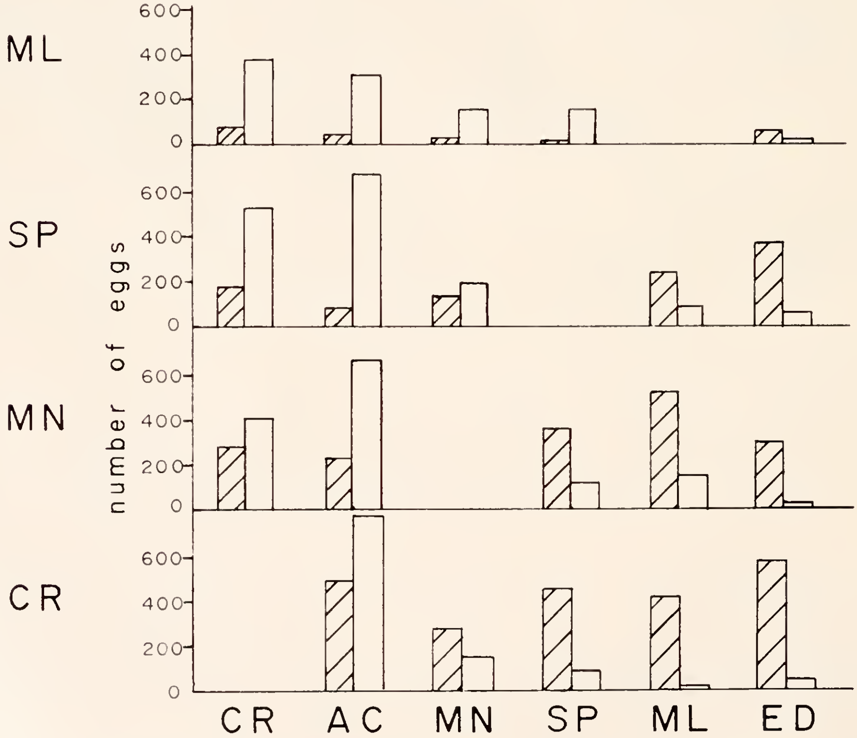

FIGURE 2. Results of adult ovipositional choice experiments. Host-plant species appear on the ordinate, test plants on the abscissa. Shaded bars indicate the number of eggs deposited on the host-plant; open bars, the number of eggs deposited on the test plant. No data are available for the ED and AC host-plants, because of high mortality in butterflies raised on those species. The members of each pair of bars are significantly different from each other as calculated by chi-square, test for goodness-of-fit ($P < 0.001$). CR represents *Passiflora caerulea;* AC, *P. alato-caerulea;* MN, *P. manicata;* SP, *P.* sp.; ML, *P. mollissima;* and ED, *P. edulis.*

TABLE I

Number of eggs laid by Agraulis *females in ovipositional choice experiments (see Figure 1) involving their host-plant species and one of the other five species of* Passiflora. *Each number represents the average mean value for the number of eggs laid per female in each experimental replicate. Each experiment involved fifty females in at least three replicates, each 48 hr long. The standard error was calculated for the replicates within each experiment. The values for tests involving AC and/or CR in each culture are larger than the next greatest value in that culture (not AC or CR) as indicated by analysis of variance ($P < 0.05$) and tests for least significant difference. AC represents* P. alatocaerulea; *CR,* P. caerulea; *MN,* P. manicata; *SP,* P. *sp.*; *ML,* P. mollissima; *and ED,* P. edulis.

Adults raised as larvae on	Mean number of eggs/female in tests involving					
	AC	CR	MN	SP	ML	ED
CR	26.09 ± 9.15	—	9.08 ± 4.7	11.47 ± 12.3	9.1 ± 5.17	12.98 ± 7.04
MN	18.81 ± 4.13	14.62 ± 2.63	—	8.65 ± 3.29	13.69 ± 2.43	7.13 ± 5.65
SP	16.05 ± 7.76	13.76 ± 4.15	7.18 ± 1.76	—	6.31 ± 0.15	8.69 ± 2.97
ML	7.14 ± 2.14	9.69 ± 6.15	3.45 ± 0.93	3.61 ± 2.3	—	1.62 ± 0.56

which both CR- and SP-raised larvae were sensitive, and that SP contained some compound(s) that accelerated feeding only in larvae raised on SP (Copp, 1976).

Adult ovipositional choice experiments

In contrast to feeding larvae, ovipositing females made highly significant choices in all experimental situations (Fig. 2). These choices did not vary with larval food plant or food preference. Cross-matching the results yields an ovipositional preference "spectrum" in which AC is the species most preferred, regardless of larval food plant, followed by CR, MN, SP, and ED in that order. Sufficient data to determine preferences were lacking for the AC culture because of high larval mortality and the very low rate of oviposition by the few emergent females.

The ovipositional choices exhibited by females in the laboratory may not be simply relative preferences. Comparing the mean number of eggs deposited per female in each choice situation (Table I) reveals that for any single culture the mean value is significantly greater in those tests with either AC (the tests for the CR, SP, and MN cultures) or CR (the ML culture tests) as one of the choices than in tests with any of the other four species. Furthermore very few eggs were deposited in the ML *vs.* ED situation. These observations suggest that the ovipositional preferences for AC and CR may result in part from the tendency of females to ovipost at a greater rate in the presence of those vine species, and that the low position of ML and ED in the preference spectrum is due to their apparent lack of any such stimulatory effect. In the field, then, where ovipositing females are presumably not often faced with a single choice situation between two species of *Passiflora* within a few feet of each other, AC and CR would still be more oviposited upon than the other four species investigated, and ML and ED would be oviposted upon very little.

Three factors contributed to some distortion in the ovipositional choice experiments: first, females were confined in a small space with vine, such that, barring very large inhibitory effects, the less preferred vine species probably received

TABLE II

Record of growth and pupation of Agraulis *larvae raised exclusively on one of six species of* Passiflora. *The data represent the combined totals of two experiments. P50 is the time in days between hatching and formation of 50% of the chrysalids, and E50 is the time in days between P50 and the emergence of 50% of the adults. Total % M is the percentage of larval and pupal mortality. FI is the fecal index (g frass deposited per larva per day). The standard error was calculated for the two experiments. CR represents* P. caerulea; *AC,* P. alato-caerulea; *MN,* P. manicata; *SP,* P. *sp.*; *ML,* P. mollissima; *and ED,* P. edulis.

Species of *Passiflora*	No. of larvae	No. of chrysalids	P50 (Days)	Number Emerged	E50 (Days)	Total %M	FI
AC	60	20	21.3 ± 0.4	15	7.3 ± 0.4	75.0	0.023 ± 0.003
CR	60	55	12.3 ± 0.3	50	7.5 ± 0.7	16.7	0.058 ± 0.014
MN	60	54	11.8 ± 0.3	42	7.5 ± 0.2	30.0	0.052 ± 0.008
SP	60	54	11.8 ± 0.9	51	8.0 ± 0.3	15.0	0.051 ± 0.001
ML	60	32	17.0 ± 1.4	25	8.3 ± 1.1	58.3	0.039 ± 0.010
ED	60	1	25.0	1	8.5	98.3	0.018

more eggs than would be expected under field conditions; secondly, as a result of the close proximity of the vine species during a test (approx. 25 cm) a female, after landing on a specimen of one species of vine which stimulates oviposition, may have moved occasionally to the other vine species before ovipositing; and thirdly, some oviposition occurred in cages devoid of passion-vine. This phenomenon may result from inadvertant selection for such behavior in laboratory cultures of insects (Wearing, Connor and Ambler, 1973). These three factors may have biased the data in favor of the less preferred vine species but are not considered significant enough to interfere with the overall interpretations.

Suitability of Passiflora *as a food plant*

This survey was conducted first from May to June, 1975, and repeated from January to February, 1976. Since the results of the second survey did not differ significantly from the results of the first, the two sets of results are combined and presented together (Tables II and III).

Larvae fed on CR, SP, and MN grew to pupation at more or less equally high rates followed by ML-, AC-, and ED-raised larvae, in that order. Mortality of larvae and pupae followed a similar pattern across the vine species; it was lowest in the CR and SP cultures and progressively higher in the MN, ML, AC, and ED cultures.

The low rate of larval maturation and high larval and pupal mortality evident in the AC, ML, and ED cultures correlate with low rates of larval frass production in these cultures (Table II). Since the rate at which frass is produced may indicate the rate at which food is consumed (Mathavan and Pandian, 1974), these results suggest that leaves of AC, ML, and ED possess factors which inhibit feeding by larval specimens of *Agraulis*. However, the possibility that the differences in the rate of frass production among the various cultures resulted from differing percentages of food utilization cannot be excluded without further experimentation.

The emergent adults in each culture were compared by measuring the length of a forewing. In some other butterfly species it has been shown that wing length is correlated with the amount of food assimilated during larval instars, and that those adults with larger wings tend to enjoy greater mating success (Wiklund, 1973). Mean wing lengths for both males and females were about equally large in the CR, MN, and SP cultures (Table III) and smaller in the other three cultures. Females were slightly, but significantly, larger than males in the CR and SP cultures and slightly smaller than males in the ML culture. No sexual dimorphism in wing length appeared in the other three cultures. One very small female emerged in the ED culture. It was mated with a male from the CR culture but died before ovipositing.

Unexpectedly, the number of eggs laid by females in each culture did not consistently follow predictions based on adult size (Table III). Unlike butterflies in the genus *Heliconius* which extract amino acids from pollen (Gilbert, 1972), *Agraulis* has no external source of amino acids aside from those present in nectar (Baker and Baker, 1973; Benson, Universidade Federal do Rio de Janiero, personal communication). Butterflies such as *Agraulis* must depend on reserves stored during larval instars for proteinaceous material used in egg production (Engleman, 1970). Consequently, low rates of larval feeding should result in small females that lack sufficient protein reserves for extensive egg production. Females from the AC culture were among the smallest produced and, indeed, they laid relatively few eggs (AC < CR, $P < 0.001$; AC < ML, MN, and SP, $P < 0.01$; chi-squared test for goodness-of-fit; Table III). Contrary to predictions, the small females from the ML culture deposited about as many eggs as the larger females from the MN and SP cultures. Possibly, females in the ML culture had assimilated sufficient protein reserves for oviposition as a result of feeding as larvae for a longer period of time, albeit at a lower rate, than larvae in the MN and SP cultures.

In general, for each of the parameters measured, *Agraulis* developed and reproduced as well or better on CR as on any of the other five species of

TABLE III

Record of adult Agraulis *raised as larvae exclusively on one species of* Passiflora. *These data represent the combined totals of two experiments. The longest dimension of a forewing was taken as a measure of adult size. P indicates probability that there is a significant difference between the mean size of females and males from a single culture (calculated by Student's* t*-test; P > 0.05; n.s., not significant). The standard error for the mean number of eggs was calculated for the two replicate experiments. CR represents* P. caerulea; *AC*, P. alato-caerulea; *MN*, P. manicata; *SP*, P. *sp*,; *ML*, P. mollissima; *and ED*, P. edulis.

Adults raised as larvae on	Number of adults	Wing length (mm)		P	Mean number of eggs laid per female
		Males	Females		
AC	15	34.2 ± 2.9	34.3 ± 3.8	n.s.	161.1 ± 69.3
CR	50	35.7 ± 2.4	38.1 ± 2.4	$P < 0.001$	300.0 ± 66.2
MN	42	36.8 ± 1.4	37.8 ± 1.7	n.s.	262.6 ± 51.6
SP	51	36.9 ± 2.6	38.7 ± 2.3	$P < 0.05$	232.6 ± 65.9
ML	25	34.9 ± 2.3	33.6 ± 1.8	$P < 0.05$	248.8 ± 6.7
ED	1	(none)	32.0		0

TABLE IV

Results of the survey of Passiflora *infestation by* Agraulis. *Each vine was sampled once every three weeks for three months during the summer of 1974. At each sampling the number of* Agraulis *eggs, larvae, pupae and adults was counted. The infestation level (mean number of* Agraulis *per* m^3*) for AC is significantly greater than for the other vines (*$P < 0.05$*; Student's* t*-test). AC represents* P alato-caerulea; *CR,* P. caerulea; *MN,* P. manicata; *SP,* P. sp.; *ML,* P. mollissima; *and ED,* P. edulis.

Species of *Passiflora*	Number of vines sampled	Mean size of vine (m^3)	Mean number of *Agraulis*/m^3
AC	28	2.66 ± 4.34	53.05 ± 32.1
CR	7	3.0 ± 2.91	10.84 ± 8.51
MN	5	3.5 ± 2.54	13.85 ± 12.34
SP	1	1.36	5.15
ML	2	13.6 ± 9.62	0
ED	2	9.64 ± 2.88	0

Passiflora tested. Larvae also grew and developed well on MN and SP, but larval mortality was higher in the MN culture and females laid fewer eggs in the SP culture than in the CR culture ($P < 0.001$, chi-squared test for goodness-of-fit). Larvae fed on ML, AC, and ED grew at a reduced rate with high mortality and produced small adults.

Field survey of Passiflora *infestation*

Based on the foregoing experimental results, it was predicted that CR would be the most infested species because it is highly preferred for oviposition and highly suitable for the growth and development of *Agraulis.* According to these indices, MN and SP should be the second most infested (both support larval development but neither is as preferred for oviposition as CR) followed by AC (highly preferred for oviposition but poor for growth and development). The remaining two species, ED and ML, should not become very infested in the field since few eggs are deposited on either in the laboratory.

These predicted relative levels of infestation were arrived at by considering that the larval feeding behavior would have no direct effect on host selection (passion-vines are generally too widely spaced for successful larval immigration; personal observation), and that the effects of larval mortality and adult oviposition would be of approximately equal importance and either balance or compliment each other. The results suggest that this is not the case (Table IV). The infestation levels follow closely a pattern that would be predicted on the basis of ovipositional preferences alone, with the exception that CR did not become more infested than MN.

Considering the unequal number of specimens available for each species of vine, and possibilities of local variations in parasitic load on *Agraulis,* inferences based on these results concerning the relation of larval and adult influences on host selection must remain tentative at best. However, in the case of ML it is likely that its failure to stimulate a high level of oviposition is responsible for the total lack of infestation by *Agraulis* since both ML vines sampled grew within a few meters of other, heavily infested passion-vines, and ML can support larval growth.

Discussion

The experimental results reported here indicate that *Agraulis vanillae incarnata* (Riley) in Santa Barbara infests its host-plants in the genus *Passiflora* primarily to the degree that each species of vine stimulates oviposition by the female butterflies. However, other, untested factors may influence patterns of host-plant infestation by *Agraulis*, such as the geographical distribution of adult butterfly nectar resources (Gilbert and Singer, 1973) and butterfly predators.

Larval feeding behavior plays a very minor role, at best, in host-plant selection by *Agraulis*. Larval immigration between host-plants is considered highly unlikely given the usually great distances between passion-vines. Consequently, larval feeding preferences could not directly affect host-plant selection (Dethier, 1959). However, induced larval feeding preferences may affect the infestation level of a particular host-plant if such preferences reduce the rate at which larvae wander from the plant and perish. This may explain why MN became as infested as CR even though the former species is less preferred for oviposition.

The feeding experiences of *Agraulis* larvae did not indirectly affect host-plant selection by influencing the ovipositional preferences of the subsequent females as predicted by Hopkins' host selection principle. Given that adaptation to a host-plant species entails a decreased capacity on the part of the insect to grow and develop on a variety of other nonhost species (Gordon, 1961), it is difficult to imagine that the mechanism of Hopkins' host selection principle, whereby larval feeding experience is transferred to the ovipositional preference of subsequent adult females, would be selected for in any but the most polyphagous species of insect.

Rather than invoke Hopkins' host selection principle to explain the origin of specific insect-plant associations, it seems reasonable to suppose that adult ovipositional preferences are genetically determined and evolve in accordance with the survival of the larvae on various potential host-plant species, as proposed for *Papilio machaon* by Wiklund (1975). *Agraulis* females would thus be expected to oviposit preferentially on those species of *Passiflora* most conducive to larval growth and development. In a broad sense, this expectation was realized but with a few notable exceptions. Two of the three most preferred species for oviposition, CR and MN, were also highly suitable as larval food plants, and the two species least preferred for oviposition, ML and ED, were among those least favorable for larval growth. In contrast, AC was the most preferred species for oviposition in the laboratory and the most infested species in the field, yet the indicators of host-plant suitability suggest that AC is one of the least suitable food plants for *Agraulis* larvae. A possible explanation for this disparity is that larvae in the field may feed on older, possibly less toxic, foliage than they were provided in the laboratory. In this way the larvae of *Eueides isabella* avoid toxins in young leaves of *P. serratifolia* (Benson *et al.*, 1975). Alternatively, if, as suspected, the ovipositional preference for AC observed in the laboratory has an absolute significance in the field, then more eggs would be deposited on that species of vine than on any other, possibly enough to compensate for a high rate of mortality.

Another inconsistency with the above prediction is that ML and SP, each apparently more suitable as a larval food plant than AC, were so little oviposited upon. The explanation may be found in the context of the ongoing reciprocal interactions intrinsic in coevolutionary relationships: ML and SP may have temporarily "escaped" heavy *Agraulis* predation pressure by virtue of their lack of oviposition stimulants.

Alternatively, these inconsistencies may be interpreted as evidence that a more static relationship is still in the process of formation. This is a distinct possibility, since passion-vines were not imported into Santa Barbara County until about 1900. *Agraulis*, presumably, was either transported along with the vine or followed in a gradual northward extension of its range. Considering the relatively short generation time of *Agraulis* (approx. 1 month under laboratory conditions), considerable adaptation to the local flora could have occurred in the past eighty years. No doubt this is the case, since the only local species of *Passiflora* that occurs in the records of host-plants for *Agraulis* in the tropics is *P. edulis* (Benson *et al.*, 1975), a species not infested locally. However, the local species of *Passiflora* probably do not accurately reflect adaptive responses to predation by *Agraulis*, since they are subject to extensive human manipulation.

In spite of these qualifications, the important role of the ovipositional preferences of *Agraulis* in determining levels of *Passiflora* infestation is probably a basic element of their association that has been carried over from its tropical origin.

We believe that, although this somewhat artificial system has not provided great insight into the course of heliconian-*Passiflora* coevolution in tropical America, its apparent simplicity has offered us a unique opportunity to investigate the behavioral control of such an association isolated from the complexities of tropical ecosystems.

We sincerely thank Mr. Greg Lee for all of his help in conducting the field study and the experiments on food plant suitability. We especially appreciate the help and support of Joyce Horanic throughout all phases of this project. Of course we are deeply indebted to Mr. Harrison Allen, Mrs. Christine Laird, Dr. Robert Haller, and all the other kind people in Santa Barbara County who so willingly allowed us, and *Agraulis*, to use their passion-vines in this research.

SUMMARY

1. Prior to an investigation of the sensory modalities and behavior involved in host-plant selection by *Agraulis vanillae incarnata* (Riley), experiments were conducted to evaluate the relative influence of larval feeding behavior, adult ovipositional behavior, and host-specific butterfly mortality in determining the infestation of various species of host-plant in the genus *Passiflora*.

2. The larvae demonstrated induced feeding preferences for some, but not all, species of *Passiflora* upon which they had previously been raised.

3. The adult females exhibited definite, fixed ovipositional preferences among six species of *Passiflora* available in Santa Barbara County. These preferences were not affected by either larval diet or induced feeding preference.

4. The food plant suitability of the six local species of *Passiflora* varied con-

siderably as indicated by several aspects of *Agraulis* growth, development, and reproduction. With a few notable exceptions this food plant suitability broadly correlated with the adult ovipositional preferences as predicted by Wiklund's model for the evolution of ovipositional preferences. The exceptions are discussed in terms of what they may reveal about the development of the *Agraulis-Passiflora* association in southern California.

5. The results of a field survey of host-plant infestation by *Agraulis* did not entirely fit predictions based on the above experimental results. The levels of infestation of the various species of *Passiflora* appear to correlate more closely with the ovipositional preferences of the adult females than with any of the other parameters studied.

6. The ovipositing females primarily determine the degree to which each species of *Passiflora* in Santa Barbara County becomes infested. The influence of larval behavior is negligible. The distribution of *Agraulis* among its host-plants is the result of an active process and is not, except in an evolutionary sense, determined by passive factors such as mortality on unsuitable food plants.

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AGRAULIS AND *PASSIFLORA* II. BEHAVIOR AND SENSORY MODALITIES[1]

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In a previous paper (Copp and Davenport, 1978) we described for the heliconian *Agraulis vanillae incarnata* (Riley) experiments on larval feeding preferences, adult ovipositional preferences, food-plant suitability of various species of *Passiflora*, and a field survey of passion-vine infestation in Santa Barbara County, California. The results indicated that, more than any other factor investigated, ovipositional preferences determine the infestation levels of the various local species of *Passiflora*. All remarks to follow concerning these aspects of the *Agraulis-Passiflora* relationship refer to the paper cited above.

If the interface of most direct interaction between the two organisms is the selection of specific passion-vines as larval food, it follows that the sensory modalities and consequent behavior governing this selection can be expected to have important effects on the evolution of both butterfly and plant. The following experiments were designed to investigate the modalities and behavior. Results are discussed in the light of the above-cited field studies.

MATERIALS AND METHODS

The butterflies and plants

All procedures for raising *Agraulis* and collecting *Passiflora* plant material have been discussed in the preceding paper. "Host-plant" was defined as that species of *Passiflora* on which the adult butterflies had been raised as larvae. *Passiflora alato-caerulea* is hereafter referred to as AC; *P. caerulea* as CR; *P. manicata* as MN; *Passiflora* sp. as SP; *P. mollissima* as ML; and *P. edulis* as ED.

Visual selection of ovipositional sites

It has been suggested that *Heliconius* females select species of *Passiflora* for oviposition on the basis of leaf shape (Gilbert, 1975). The possibility that specific visual cues might function in the ovipositional preferences of *Agraulis* was tested in the following manner.

A single new-growth shoot of a passion-vine (*ca.* 5 leaves with the cut end in 10 ml water) was placed inside an inverted one-gallon glass container fitted with an air-tight lid. Four, 2.5 cm-wide strips of cheesecloth, taped to the top and sides of the container, facilitated landing and oviposition by the female butterflies.

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[1] This work was supported by National Science Foundation Grant GB 41210.

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This was placed in a cage (61 cm square, 90 cm tall) beside an identical container either left empty or containing a new-growth shoot from a different species of plant. In experiments involving two species of vine, the appropriate removal of leaves equalized the surface area presented by each.

An experimental trial consisted of placing two containers, comprising an experimental pairing, in a cage with females (four days old, mated, and raised as larvae on either SP or CR), reversing their position after 24 hours, and counting the number of eggs on each after 48 hours. Each experimental pairing was conducted three times under laboratory culture conditions (23° C, 60% humidity; 15L:9D).

Three different plant species were used in these experiments: AC, the vine most oviposited upon by *Agraulis* in laboratory preference tests; ED, the vine least oviposited upon in ovipositional preference tests; and *Hedera canariensis* (Algerian Ivy), a readily available plant not oviposited upon by *Agraulis* in the laboratory. Leaves of these three plants differ sufficiently in both color and shape to make them easily distinguishable by the human observer. In a fourth experimental pairing a container with leaf models, cut from green construction paper to the shape of AC leaves, was paired with an empty control container.

The butterflies had had no previous exposure to plant material prior to all experiments except those matching AC with *Hedera*. In the excepted case, four-day old females were allowed to oviposit on sprigs of AC and *Hedera* in the laboratory for an additional four days immediately preceding the test. The increased age of the females in this test probably had no effect on the results since oviposition behavior has not been observed to change during the period of active oviposition in a female's life span.

Volatile stimulants of oviposition

Observations of females in the laboratory suggested that a volatile agent, emitted by some species of *Passiflora*, accelerated oviposition by *Agraulis* females. The following experiment examines the effect on ovipositional behavior of substances volatilized from *Passiflora* leaves.

A three-gallon glass container with an air-tight lid was equipped with a 500 ml Nalgene bottle and a wire screen cylinder such that females placed in the glass container were able to "smell" vine enclosed in the Nalgene bottle but neither make contact with it nor see it. About 150 holes, each 1.5 mm in diameter, were drilled in the side of the Nalgene bottle, and its lid was attached with white glue to the inside of the lid of the glass container. A wire screen cylinder (10.5 cm in diameter, 21 cm tall), closed at the bottom, fitted into the neck of the glass container and prevented tarsal and proboscis contact by the butterfly with the vine contained inside the Nalgene bottle. Odors could circulate freely.

Prior to an experiment, ten mated, four-day old females were isolated from their cages and fed a 1 M sucrose solution. Five females were then placed in each of two glass containers (one experimental, one control), the wire cylinders inserted, and the containers' lids screwed down, thus suspending the Nalgene bottles within the wire cylinders. Ten 20 cm long sprigs of fresh new-growth shoots of *Passiflora* (50 leaves), placed with water in the Nalgene bottle, provided the olfactory stimulus. Females oviposited primarily on a circle of filter paper placed on the floor of the glass container. Experimental containers with vine and control containers without vine were placed under the cage lights in the culture laboratory, left 24 hours, reversed, and left another 24 hours. The number of eggs in each was counted after 48 hours. Each experimental situation was replicated six times involving a total of 60 females.

Washing all pieces of equipment with 50% ethanol after each experimental replicate and randomizing their assembly helped to control for residual, adsorbed odors. Presumably, humidity levels did not differ between paired bottles. Thermometers, taped to the inside of the glass containers, indicated that in all cases both experimental and control containers became equally warm over the course of the experiment (30° C).

Anemotactic behavior

The ease with which a potential host-plant is located by gravid *Agraulis* females will partilly determine that vine's level of infestation, assuming that females spend an appreciable amount of time searching for ovipositional sites. It has long been known that some adult lepidopterans exhibit a positive anemotaxis (directed movement upwind) when stimulated by certain olfactory agents such as sex pheromones. The possibility that *Passiflora* odors elicit a positive anemotaxis from *Agraulis* females was tested in the following experiments.

A four- to six-day old female was tethered in the following manner to allow restricted flight in any direction: one end of a length of light weight thread was attached to the butterfly's thorax with a small piece of bubble-gum (a biologically inert adhesive with immediate "stickiness," low price, and recreational value) and the other end passed through a 15 cm length of small diameter glass tubing clamped vertically to a ring stand. The butterfly's thorax was drawn up within 5 cm of the glass tube allowing that much forward flight and unrestricted turning.

The tethered insect could then be subjected to a slow airstream (*ca.* 0.9 m/sec) created by an electric fan, the speed of which was regulated by a Powerstat variable autotransformer. The fan, mounted 0.66 meters from the butterfly, directed air over a group of plant shoots (about 200 leaves) placed beneath the butterfly's visual field. A square card, divided into four equal quadrants, was taped to the table directly beneath the suspended butterfly. A line bisecting one of the quadrants pointed towards the center of the fan. Forty-five degrees either side of this line was defined as the "upwind" direction. The orientation of a butterfly in stationary flight could then be monitored by noting, from above, the quadrant within which the extended tether-string fell. This apparatus was placed in in a small room separate from the culture laboratory but under the same temperature and humidity conditions (23° C, 60% humidity).

Twenty-four hours prior to an experiment the females to be tested were selected from their cages, with no preference for host-plant, and placed without vine in a holding cage in the separate test laboratory. Just before bringing vine into this room all females were further isolated in air-tight, one-gallon glass containers, five females per container, to minimize their exposure to vine odors prior to testing. A female, selected from one of the containers and attached to the tether, stood on a small platform for one minute. The fan was turned on at the end of this period and the platform removed. A single trial began with the consequent, reflex onset of flight. The butterfly was randomly oriented with respect to the direction of the airstream at that time. Each trial lasted for five minutes during which the amount of time that the tether pointed in the upwind direction was recorded with a stopwatch. All experiments began at 10:00 AM, four hours after the butterfly's "dawn." Thirty females, in blocks of ten, comprised three replicates of both experimental and control situations. Only those females that flew the entire five minutes were recorded, and each flew only once.

Specimens of AC, MN, and ML were used as odor sources because they represent high, median and low points in the spectrum of ovipositional preferences and in observed levels of infestation in the field. Control experiments omitted the vine or substituted *Hedera canariensis* for *Passiflora*.

Because the laboratory became contaminated with plant odors during the experimental trials, it was not possible to subject one butterfly to a control trial immediately followed by an experimental trial on the same butterfly and still accumulate an adequate sample size in a reasonable amount of time. Control and experimental trials occurred several days apart and involved different groups of equally old females.

Dispersal of adult Agraulis *with respect to host-plants*

The relative significance of visual cues, volatile stimulants and inhibitors of oviposition, and olfactory orientation to host-plants in determining the level of infestation of various species of *Passiflora* by *Agraulis* will vary with the degree of active host seeking and dispersal of the ovipositing females. The movements of adult butterflies among several separate passion-vines were observed during a mark-release-recapture study conducted in Isla Vista, California in the summer of 1975.

All captures of butterflies occurred at one of three passion-vines which formed a triangle (site I to site II, 385 m; I–III, 220 m; II–III, 325 m). No other passion-vines existed within or near this triangle to our knowledge. Prevailing wind was from WSW (*i.e.*, approximately) from site I (MN vine) toward sites II and III (AC vines). An observer stood near a vine and captured as many *Agraulis* butterflies as possible within a one meter radius of the vine for a period of one hour in the late morning or early afternoon. Butterflies were individually marked by writing a number on the underside of the wings with a "Sharpie" laundry marker. Numbering was easy, permanent and permitted recording of an individual's movements among the three vines. When released, the numbered butterflies flew away immediately and showed no ill effects as a result of the handling. The number, sex, and time of capture for each butterfly was recorded according to location.

Each of the three locations was sampled every day for the first two weeks beginning July 10, 1975 and three times a week thereafter until October 13, 1975. The three vines were always sampled consecutively on the same day. The order of their sampling was random. At each sampling the observer recorded the marks of all recaptured butterflies (those numbered prior to the sampling). Multiple

TABLE I

Results of tests for visual discrimination of different species of plants by ovipositing Agraulis. *Each experimental trial lasted 48 hours. P was calculated by Student's* t-test *for paired comparisons* ($P > 0.05$; *n.s., not significant*). *AC represents* Passiflora alato-caerulea; *ED,* P. edulis; *and* Hedera, Hedera canariensis.

Experimental pairing	Trial number	Number of females	Number of eggs	P
AC/blank	1	32	97/ 16	
	2	10	59/ 19	
	3	17	110/ 38	
	total	59	266/ 73	$0.01 < P < 0.02$
AC/ED	1	31	47/ 15	
	2	31	23/ 26	
	3	39	102/133	
	total	101	172/174	n.s.
AC/*Hedera*	1	30	70/ 44	
	2	31	35/ 43	
	3	25	25/ 88	
	total	86	130/175	n.s.
leaf model/blank	1	20	77/ 18	
	2	10	52/ 3	
	3	10	59/ 1	
	total	40	188/ 22	$0.002 < P < 0.01$

recaptures of a single butterfly in one day were recorded for each event, but only the first recapture on that day entered into the calculation of movement patterns unless they occurred at different sites.

RESULTS

Visual selection of ovipositional sites

The results of the visual selection experiments are presented in Table I. The container with AC vine and the one with the green paper leaf model were both more oviposited upon than the empty control containers. The AC container did not receive significantly more eggs than either the ED or the *Hedera* container.

Prior to the third experimental pairing, females had been allowed to oviposit on fresh sprigs of AC and *Hedera;* during this time the latter plant received no eggs, while AC was heavily oviposited upon. After this 4-day period of freedom of access the same females were given the same choice in the absence of chemical cues; under such circumstances they made no significant choice. Apparently, as the results of this test show, the females did not, in the time available, learn to associate leaf color and shape with a suitable ovipositional stimulus.

Table II

Effects of Passiflora *odors on oviposition by* Agraulis. *The number of eggs is the total number laid by thirty females during six replicate experiments. In each replicate, a group of ten females was divided equally between two containers; one with* Passiflora, *and one without (blank). P was determined by Student's* t*-test for paired comparisons ($P > 0.05$; n.s., not significant). AC represents* P. alatocaerulea; *CR,* P. caerulea; *MN,* P. manicata; *SP,* P. *sp.*; *ML,* P. mollissima; *and ED,* P. edulis.

Experimental pairing	Number of eggs	P
AC	130	$P < 0.05$
Blank	37	
CR	955	$P < 0.05$
Blank	570	
MN	314	$P < 0.05$
Blank	464	
SP	308	$P < 0.05$
Blank	506	
ML	381	n.s.
Blank	372	
ED	116	n.s.
Blank	129	

In preliminary experiments (Copp, 1976) which monitored oviposition when passion-vine (AC) either was or was not visible to the females, two additional points pertinent to the role of visual cues in oviposition were revealed: first, when direct contact was prevented, eggs were laid adjacent to passion-vine only when the vine could be seen; and secondly, maximal rates of oviposition occurred only when females had full access (visual, olfactory, and tactile) to the vine.

All of these results indicate that ovipositing *Agraulis* females preferentially localized their eggs on containers with green, leaf-shaped objects but did not visually prefer one species of *Passiflora* over another, nor did they visually select a *Passiflora* over the nonpassifloraceous plant, *Hedera*. Large numbers of eggs were even deposited on a container with crude paper leaf models inside. Apparently, visual cues are important as generalized attractants of ovipositing females rather than as indicators of specific host-plant species.

Volatile stimulants of oviposition

Although the replicates of each experimental situation occurred within two weeks of each other and involved females of similar history, different situations were tested at different times of the year with females raised as larvae on different species of *Passiflora*. This prevents extensive comparison between experimental situations. However, the results (Table II) indicate that: AC and CR emitted some factor which significantly increased the rate of oviposition in those containers over the controls; ML and ED odors had no effect on the rate of oviposition; and odors emitted by MN and SP inhibited oviposition slightly. The unexpectedly high rates of oviposition in most of the control containers may

TABLE III

Amount of time out of five minutes that tethered Agraulis *females faced upwind in airstreams labeled with various plant odors. Mean time represents the mean upwind flight time for thirty females. P was calculated by Student's* t*-test comparing the mean with a hypothetical value of* $\mu_0 = 75$ *sec* ($P > 0.05$; *n.s., not significant). The standard error of the mean is given in seconds. AC represents* Passiflora alato-caerulea; *MN,* P. manicata; *ML,* P. mollissima; *and* Hedera, Hedera canariensis.

Airstream treatment	Mean upwind flight time/5 min (min:secs)	P
Unlabeled	1:00 ± 6.2	$P > 0.05$
AC	2:23 ± 11.5	$P < 0.001$
MN	2:21 ± 16.2	$P < 0.001$
ML	1:12 ± 10.0	n.s.
Hedera	1:13 ± 10.4	n.s.

have resulted from inadvertent selection for spontaneous oviposition in laboratory cultures of insects as previously discussed (Copp and Davenport, 1978).

In the ovipositional preference tests the mean number of eggs laid per female was highest in those experiments which included either AC or CR as one of the choices. This phenomenon may now be explained by the presence in those tests of volatile stimulants of oviposition released by AC and CR.

The effects of volatile compounds, as noted in these experiments, cannot account entirely for the degree of resolution of host-plant species evident in the ovipositional preference experiments. Specimens of MN and SP for example, were consistently more oviposited upon than either ML or ED, yet the former two species emitted a volatile inhibitor of oviposition not present in ML and ED leaves. Also, as mentioned above, maximal rates of oviposition occurred only when the females were free to contact the vine. It seems likely that contact-chemical cues, in addition to volatile chemical signals, function in determining ovipositional preferences. This hypothesis is supported by observations that female specimens of *Agraulis* frequently "drum" a leaf's surface with their foretarsi just before ovipositing. Drumming is a behavioral act thought to allow "tasting" of a leaf's chemical constituents by the female butterfly (Ilse, 1937; Fox, 1966; Ma and Schoonhoven, 1973). Numerous attempts were made to develop an effective bioassay for the detection of these presumed contact-chemical cues in *Passiflora* leaves, but with no conclusive success to date. The chromatographic "spot" patterns of extracts from fresh *Passiflora* leaves have been compared. No correlations with ovipositional preference have been observed. Differences in leaf texture among the six species of *Passiflora* studied appeared minimal and generalized, and were not considered to have a significant influence on specific ovipositional preferences.

Anemotactic behavior

For a female butterfly which is orienting randomly with respect to the direction of the airstream, the upwind flight time should not differ significantly from 1 minute and 15 seconds, *i.e.*, one quarter of the trial period. Significantly non-

TABLE IV

Mark-release-recapture data for Agraulis *in Isla Vista, California, July 10 to October 13, 1975. Site I is a MN vine; sites II and III are AC vines. The "mark site" is the site at which the butterfly was originally marked.*

Site	Number of butterflies marked	Number recaptured at least once	Total number of recaptures	Number recaptures	
				At mark site	At other sites
I	203	127	337	296 (87.8%)	41
II	120	82	188	180 (95.7%)	8
III	39	17	36	29 (80.6%)	7

random upwind orientation by *Agraulis* females occurred only when the airstream had passed over either AC or MN vine (Table III). Females did not face preferentially upwind into an unlabeled airstream or ones which had passed over either *Hedera canariensis* or ML vine. Indeed, females exhibited a slight tendency to face down- or across-wind in an unlabeled airstream. Variations in the age of females or their sensitivity to plant odors may have caused the high variability in the experiments involving AC and MN.

Although olfactory cues have been shown to be involved in the localization of host-plants by insects in other orders (*e.g.,* Moorehouse, 1971; Werner, 1972), this may be the first experimental demonstration of such a capability in a lepidopteran. The results of Yamamoto and Fraenkel (1960), Schurr and Holdaway (1970), and Wearing, Connor and Ambler (1973), which may indicate chemically triggered orientation by lepidopterans, are open to other interpretations.

Dispersal of adult butterflies with respect to host-plants

Monitoring the movements of *Agraulis* among three passion-vines revealed that the adults remain highly localized with respect to larval food resources (Table IV). Despite observations that *Agraulis* is a strong flier, lives up to four weeks in the field, and forages widely for nectar, there was little movement of adults among vines 220 to 385 meters apart.

The problem of correcting for immigration, birth, and death prevented accurate estimates of population size. Hereafter the apparent size of a subpopulation of *Agraulis* refers to the number of butterflies captured at a particular site over the four month period.

To test whether the butterflies that were repeatedly recaptured at any one of the three vines sampled consisted of adults which had emerged on or near that vine, 40 pupae (36 at site II, 4 at site I) were enclosed in loose cheesecloth sacks and the adults marked upon emergence. All but eight died prior to marking, mostly as a result of tachinid fly parasitization, but also as a result of incomplete emergence. The recapture records of the few surviving adults support the contention that adults return repeatedly to their larval food plant's site. Of the seven adults that emerged at site II, six were recaptured a total of twelve times, each time at site II. One adult from site II was never recaptured. The one adult that emerged at site I was recaptured three times only at site I.

A more detailed look at the instances of emigration between sites reveals the following points: first, of the 41 butterflies that moved between vines at least once, 24 were female and 17 male. Males and females exhibited no significant difference in their tendencies to emigrate ($P < 0.05$, chi-squared test for goodness-of-fit); secondly, the apparent size of a site's subpopulation did not correlate with the proportion of marked butterflies that emigrated from that site as was found to be true for *Pieris* (Shapiro, 1970). Site I, a large MN vine with an apparently dense subpopulation, yielded 30 of the 41 emigrants, *i.e.,* 15.2% of the total number of butterflies marked at site I moved between sites at least once. This compares with 12.8% emigrants from site III, a small, relatively sparsely populated, AC vine, and 5.0% from site II, an AC vine of intermediate size and population density; and thirdly, emigrants did not favor any site for arrival. The 30 emigrant adults numbered at site I made 13 trips to site II, 19 to site III, and only eight return trips. The six emigrants from site II made a total of six trips, three to each of the other sites, and no return trips. Three of the five emigrants from site III went to I, two to site II and none made return trips. Considering the results of the adult flight orientation experiments and the prevailing westerly wind direction, emigrating butterflies had been expected to move upwind toward site I, but they did not do so.

DISCUSSION

The foregoing experiments indicate that the ovipositional preferences of *Agraulis,* as exhibited in the laboratory, can be explained as graded responses to various ratios of volatile and contact-chemical signals which affect oviposition. The ovipositional responses may be triggered (*e.g.,* AC, CR, MN, SP) or inhibited (*e.g.,* ML and ED) by contact-chemical stimuli. The rate of oviposition after triggering could be partially determined by volatile stimulants (*e.g.,* AC and CR) or inhibitors (*e.g.,* MN and SP). Gradations in quantity or differences in quality of either chemical component could account for finer resolution of host-plant species by ovipositing females. Pre-ovipositional behavior and oviposition in several species of Lepidoptera have been shown to be affected by both volatile cues (*e.g.,* Vaidya, 1969; Städler, 1974; Sutherland, Hutchins and Wearing, 1974) and contact-chemical cues (*e.g.,* Yamamoto, Jenkins, and McClusky, 1969; Ma and Schoonhoven, 1973).

The generalized role of vision in the selection of ovipositional sites by *Agraulis* is not surprising in view of similar findings for other lepidopterans (*e.g.,* Ilse, 1937; Vaidya, 1969; Wiklund, 1974). However, it has been proposed that *Heliconius* butterflies select species of *Passiflora* for oviposition primarily on the basis of learned or innate visual responses to specific leaf shapes (Gilbert, 1975). Our experiments gave no indication of this capability in *Agraulis.*

Considering the low frequency with which *Agraulis* butterflies move between specimens of *Passiflora,* the upwind flight response does not appear to be significant in affecting host-plant selection in the field. There are other possible functions for this orientational response: it may aid in the localization of new host-plants if, in response to extreme host-plant depletion, *Agraulis* dispersal increases. Since *Agraulis* larvae frequently pupate some distance from the host-plant, the upwind flight response may serve in the initial localization of the host-plant by the newly emergent adult. A third possible function is that it may, in effect, "trap" adults around their particular larval food plant. None of these possibilities excludes the others, and each may work in concert with visual orientation to learned landmarks. The ability of other adult heliconians to return repeatedly to a single site is evident in their "traplining" of pollen resources (Ehrlich and Gilbert, 1973).

The division of a population of butterflies into a number of subpopulations, each centered on a different patch of plant resources, has been observed in *Heliconius* (Erhlich and Gilbert, 1973) and *Euphydryas editha* (Ehrlich, 1961; Gilbert and Singer, 1973; Ehrlich and Singer, 1974). For these butterflies adult dispersal seems to depend mainly on the distribution of the adult's food resources. In contrast, subpopulations of *Agraulis* in Santa Barbara County were found to be centered on single larval food-plants. *Agraulis* in the tropical zone might be expected to disperse more widely among larval food plants as a consequence of increased parasitic pressure and competition for food, much as *Heliconius* avoids local concentrations of parasites or larval overcrowding by spreading out its eggs in both space and time (Gilbert, 1975).

In southern California it would seem that the pattern of *Passiflora* infestation does not result from active choice by female butterflies searching for ovipositional sites, as much as from the "trapping" of sedentary populations on specific plants, the levels of infestation depending primarily on the balance of compounds affecting oviposition particular to that species of *Passiflora*. The following model for local host-plant infestation by *Agraulis* is proposed. A previously uninfested passion-vine, or one which has lost its population by winter-killing, is located by a female, possibly as a consequence of an upwind flight response to host specific odors followed by a series of nonspecific, visually directed test landings. Oviposition is apparently triggered or inhibited by appropriate contact-chemical cues. If triggered, it proceeds at a rate dependent on the concentration of chemical stimulators and/or inhibitors (volatile and nonvolatile). Following successful development, subsequent generations of females oviposit on the same individual plant. The newly established subpopulation of butterflies increases at a rate which depends primarily on the rate of oviposition and, secondarily, on the rate of larval and pupal mortality on that species of vine.

The local association between *Agraulis* and *Passiflora* could be severely affected at any number of control points at each step of this proposed model. These control points need not necessarily involve plant chemistry. For example, Gilbert (1971) has demonstrated that hooked trichomes on the leaves of *Passiflora adenopoda* act as a highly effective barrier to heliconian infestation by virtue of their lethal effect on the larvae. Also, Singer (1971) has demonstrated in *Euphydryas editha* that the growth forms and microhabitats of various potential host-plant species determine which is most infested in a single locale by a particular population of the butterfly. However, the *Agraulis-Passiflora* association in Santa Barbara County appears to hinge on plant chemistry, more particularly, on these chemical factors directly involved in the butterflies' specific ovipositional responses. Plant chemistry has been shown to be an important element in the coevolutionary relationships between other lepidopterans and their host-plants (Ehrlich and Raven, 1964; Dolinger, Ehrlich, Fitch and Breedlove, 1973).

It would broaden our understanding of the coevolutionary relationship between heliconians and passion-vines to examine further the role of plant chemistry in host-plant localization and oviposition by the butterflies and to conduct an in-depth chemical survey of the family Passifloraceae from the standpoint of the chemical control of butterfly behavior.

We would like to thank Mr. Stephen Maskel whose accuracy with a butterfly net helped us considerably in the mark-release-recapture study.

Summary

1. The behaviors and sensory modalities involved in the selection of *Passiflora* host-plants for oviposition by the heliconian butterfly *Agraulis vanillae* were investigated.

2. Ovipositing females are attracted to oviposit near green leaf-shaped objects on the basis of generalized visual cues but make no ovipositional distinction between *P. alato-caerulea* and *P. edulis,* which are readily discriminated when full access to the vines is allowed.

3. The rate of oviposition by female *Agraulis* is accelerated by odors from *P. alato-caerulea* and *P. caerulea*, retarded by odors from *P. manicata* and *Passiflora* sp., and not affected by odors from *P. edulis* or *P. mollissima.*

4. Contact-chemical stimulants and inhibitors of oviposition are presumed to exist in the leaves of passion-vines on the basis of circumstantial evidence. Attempts to demonstrate directly their existence have not yet been successful.

5. Female *Agraulis* exhibit a positive anemotactic response in an airstream that has passed over one of at least two species of *Passiflora, P. alato-caerulea* and *P. manicata,* but not in an airstream passed over *P. mollissima.*

6. A mark-release-recapture study of adult movements among three passion-vines demonstrated that butterfly subpopulations tend to form on each vine with little exchange between different subpopulations.

7. The primary factor determining the infestation level of a particular *Passiflora* by *Agraulis* is the balance of chemical cues directly involved in oviposition that are present in that *Passiflora.* Possible functions for the upwind flight response are suggested. Further research on the plant chemistry of the family Passifloraceae as it relates to heliconian behavior would broaden our understanding of heliconian-*Passiflora* coevolution.

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GENETIC VARIABILITY IN DEEP-SEA ORGANISMS

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In the last few years many researchers have studied genetic variability in marine organisms, in a search for correlations between polymorphism and environmental stability. A high degree of polymorphism has been found in deep-sea organisms which indicates a high genetic variability (Schopf and Gooch, 1971; Doyle, 1972; Gooch and Schopf, 1972; Ayala and Valentine, 1974; Ayala, Valentine, Hedgecock and Barr, 1975). These data are not in agreement with the hypothesis that predicted an impoverishment of genetic variability in those populations that live in highly specialized environments (Levins, 1968; Grassle, 1972).

In these works a highly stable deep environment had always been chosen; in the present research, however, the species considered come from an area that presents a certain instability of some environmental features. This area is the Wyville-Thomson Ridge (Shetland-Faroe Channel) which is intermittently subjected to overflows of Norwegian Sea deep waters flowing in a southwest direction towards the Northern Rockall Trough (Ellet and Roberts, 1973).

Materials and Methods

Populations studied

Samples were collected in the area of the Northern Rockall Trough and the Wyville-Thomson Ridge (Northeast Atlantic Ocean) in the period from June 30, 1976 to July 13, 1976, during the scientific cruise made by the R.R.S. CHALLENGER, organized by the Scottish Marine Biological Association. The Agassiz-trawl was used for the sampling. On July 2 specimens of the crustacean species, *Munidopsis hamata,* were collected at a depth of 1331 meters along a transect from 59° 13′ 06″ N; 08° 01′ 05″ W to 59° 13′ 46″ N; 08° 00′ 58″ W. On July 7 gastropods of a species belonging to the genus *Buccinum* and echinoderms belonging to the species *Ophioglypha bullata* were collected at a depth of 1058 m on a transect extending from 60° 04′ 08″ N; 05° 56′ 80″ W to 60° 04′ 86″ N; 05° 59′ 02″ W. On July 11 the echinoderm *Ophiomusium lymani* was collected at the depth of 1900 m from 57° 06′ 08″ N; 12° 06′ 01″ W to 57° 06′ 73″ N; 12° 05′ 27″ W.

The fishing operation lasted about four hours on July 2 and 7 and about six hours on July 11. Specimens were immediately stored in the ship's deep freezer at −20° C, and in the laboratory the specimens were stored at −30° C until the time of dissection.

Gel preparation

The starch used is Sigma (Sigma Chemical Co.). A 12% (w/v) solution of starch in gel buffer was heated to near boiling point and degassed with an aspirator and then poured in a plexiglass mold, 10 × 20 × 0.3 cm (volume 100 ml). After the electrophoresis, for each run, two different stains were carried out by dividing the gel in two layers. In some cases electrophoresis was performed on acrylamide gel (from Serva feinbiochemica) on vertical slab. The methods followed in these cases have been described by Bisol, Varotto and Battaglia (1976).

Gel and electrode buffers

Six types of buffer were used (the buffers for the gel were the same used for the electrodes, diluted 1 : 1, except the buffer D diluted 1 : 10) : A, 0.083 M Tris and 0.081 M boric acid, pH 8.5; B, 0.083 M Tris and 0.048 M boric acid, pH 8.8; C, 0.01 M Tris, 0.01 M maleic acid, 0.0025 M NaOH and 0.0002 M $MgCl_2$, pH 7.4; D, 0.135 M Tris and 0.045 M citric acid, pH 7; E, 0.023 M Tris and 0.005 M citric acid, pH 8; and F, 0.076 M Tris and 0.05 M citric acid, pH 8.8.

Sample preparation

Crude protein extracts were prepared from dissected samples of tissue (soft tissue of ophiuroids, muscle and hepatopancreas of decapods, foot and visceral sac of the gastropods) by homogenization in about two volumes of Tris-HCl pH 8, 0.05 M buffer. The homogenates were centrifugated at 15,000 rpm for 10 min. Five to 10 μl of the supernatant were absorbed with a 4 × 4 mm piece of Whatman

TABLE I

Enzymes assayed and buffer systems used in the study of genetic variation in four species of deep-sea organisms.

Enzymes	Abbreviation	Buffer system*	Number of loci scored in			
			Buccinum sp.	*Ophioglypha bullata*	*Munidopsis hamata*	*Ophiomusium lymani*
Acid phosphatase	*Acph*	A	3	2	2	1
Alkaline phosphatase	*Aph*	B	7	1	1	2
Aminopeptidase	*Ap*	A	3	2	3	2
Esterase	*Est*	A	6	7	10	4
Glyceraldehyde-3-phosphate dehydrogenase	*G-3-pdh*	F	—	1	—	—
Hexokinase	*Hk*	D	—	1	1	1
Isocitrate dehydrogenase	*Idh*	C	1	1	1	1
Leucine aminopeptidase	*Lap*	A	2	2	1	2
Lactate dehydrogenase	*Ldh*	A, C	1	2	1	1
Malate dehydrogenase	*Mdh*	A	1	1	1	1
Malic enzyme	*Me*	E, F	1	—	1	1
Nothing dehydrogenase	*Ndh*	A	2	—	1	—
Phosphoglucomutase	*Pgm*	A, C	1	1	1	1
Phosphohexose isomerase	*Phi*	A, G	1	1	1	1
6-Phosphogluconate dehydrogenase	*6-Pgdh*	D	—	—	1	—
Tetrazolium oxidase	*To*	A, E	—	3	3	1

* See Materials and Methods for a description of the buffer systems.

Table II

Allelic frequencies at eleven polymorphic loci in a deep-sea population of Buccinum *sp.*

Locus	N*	Number of alleles	Allelic frequencies			Frequency of heterozygotes		Polymorphic loci**	
			a	b	c	Observed	Expected	(1)	(2)
Ap-3	44	3	0.045	0.932	0.023	0.136	0.129	+	+
Aph-4	44	3	0.250	0.045	0.705	0.227	0.439	+	+
Aph-5	44	3	0.273	0.636	0.091	0.500	0.512	+	+
Aph-6	44	3	0.114	0.636	0.250	0.636	0.520	+	+
Est-1	44	2	0.159	0.841	—	0.318	0.268	+	+
Idh-1	44	2	0.023	0.977	—	0.045	0.044	+	−
Lap-2	44	3	0.341	0.568	0.091	0.318	0.553	+	+
Mdh-1	44	2	0.886	0.114	—	0.136	0.201	+	+
Pgm-1	44	3	0.023	0.955	0.022	0.091	0.088	+	−
Phi-1	44	2	0.023	0.977	—	0.045	0.044	+	−
Me-1	44	2	0.068	0.932	—	0.136	0.127	+	+

* N: number of genes (twice number of individuals) sampled at each locus.

** Two criteria are used to decide whether a locus is polymorphic, on the basis of the frequency (P) of the most common allele: (1) $P < 0.990$; (2) $P < 0.950$. The same criterion was used in Tables III, IV, and V.

No. 3 filter paper. The soaked filter paper wicks were placed into the starch gel slab for horizontal electrophoresis. All these operations were carried out in a cold room at 4° C.

Enzyme assays

The systems were stained according to Shaw and Prasad (1970), Ayala, Powell, Tracey, Murão, and Pérez-Salas (1972) and Bisol (1976), with some modifications concerning, almost exclusively, the pH of stain buffers.

Gel fixation

After the enzyme bands appeared, the reaction was stopped by washing the gel with water and adding fixing solution: 50 parts methanol, 10 parts glacial acetic acid, and 40 parts H_2O. After fixation, the electrophoretic patterns were ready for interpretation.

RESULTS

The observed patterns have been interpreted under the assumption of Mendelian segregation in diploids (Brewer, 1970). Frequencies have been calculated on the basis of the Hardy-Weinberg principle. The nonvarying zones have been assumed to be controlled by a single locus.

Buccinum sp.

The electrophoretic analysis performed on 22 specimens of this snail for 12 enzymatic systems has revealed 29 zones of activity (Table I).

TABLE III

Allelic frequencies at thirteen polymorphic loci in a deep-sea population of Ophioglypha bullata.

Locus	N	Number of alleles	Allelic frequencies			Frequency of heterozygotes		Polymorphic loci	
			a	b	c	Observed	Expected	(1)	(2)
Ap-2	50	2	0.460	0.540	—	0.200	0.497	+	+
Aph-1	48	2	0.729	0.271	—	0.208	0.400	+	+
Est-4	42	2	0.333	0.667	—	0.286	0.444	+	+
Est-6	44	3	0.023	0.409	0.568	0.227	0.509	+	+
Hk-1	44	3	0.045	0.500	0.455	0.591	0.541	+	+
Idh-1	50	2	0.980	0.020	—	0.040	0.039	+	—
Lap-1	38	2	0.158	0.842	—	0.211	0.266	+	+
Lap-2	38	2	0.763	0.237	—	0.158	0.361	+	+
Ldh-2	68	3	0.059	0.676	0.265	0.147	0.469	+	+
Mdh-1	50	3	0.060	0.700	0.240	0.280	0.224	+	+
Pgm-1	50	2	0.640	0.360	—	0.240	0.460	+	+
Phi-1	50	2	0.640	0.360	—	0.240	0.460	+	+
To-3	50	2	0.600	0.400	—	0.320	0.480	+	+

One of these zones, leucine aminopeptidase, has not been included in the data, as it was found in only a few specimens. It did, however, show different electromorphs. Among the remaining 28 zones, 17 appeared monomorphic, while 11 were polymorphic (Table II).

Ophioglypha bullata

Twenty-five individuals of this brittle star were analyzed. Nine additional individuals have been analyzed subsequently so as to be able to interpret the lactate dehydrogenase system. Twenty-three activity zones attributable to the action of of 23 genic loci were found (Table I). Two further esterase zones, being characterized by an electrophoretic pattern of uncertain interpretation, have been omitted in the data (Table III).

TABLE IV

Allelic frequencies at seven polymorphic loci in a deep-sea population of Munidopsis hamata.

Locus	N	Number of alleles	Allelic frequencies				Frequency of heterozygotes		Polymorphic loci	
			a	b	c	d	Observed	Expected	(1)	(2)
Ap-3	44	2	0.386	0.614	—	—	0.409	0.474	+	+
Est-9	28	2	0.964	0.036	—	—	0.071	0.068	+	—
Hk-1	46	4	0.043	0.783	0.152	0.022	0.348	0.362	+	+
Idh-2	44	2	0.159	0.841	—	—	0.136	0.267	+	+
Lap-3	42	3	0.524	0.452	0.024	—	0.714	0.520	+	+
Pgm-1	38	3	0.850	0.075	0.075	—	0.100	0.266	+	+
6-Pgdh	46	2	0.696	0.304	—	—	0.435	0.423	+	+

Table V

Allelic frequencies at nine polymorphic loci in a deep-sea population of Ophiomusium lymani.

Locus	N	Number of alleles	Allelic frequencies					Frequencies of heterozygotes		Polymorphic loci	
			a	b	c	d	e	Observed	Expected	(1)	(2)
Ap-1	94	2	0.138	0.862	—	—	—	0.277	0.238	+	+
Ap-2	94	2	0.745	0.255	—	—	—	0.170	0.380	+	+
Hk-1	88	5	0.080	0.364	0.227	0.284	0.045	0.886	0.727	+	+
Idh-1	68	2	0.353	0.647	—	—	—	0.235	0.457	+	+
Ldh-1	92	3	0.826	0.152	0.022	—	—	0.261	0.294	+	+
Me-1	82	3	0.183	0.756	0.061	—	—	0.195	0.391	+	+
Pgm-1	92	5	0.087	0.185	0.413	0.283	0.033	0.543	0.701	+	+
To-1	92	4	0.054	0.402	0.261	0.283	—	0.652	0.687	+	+
Phi-1	94	2	0.926	0.074	—	—	—	0.021	0.138	+	+

Munidopsis hamata

Among the 29 zones of activity detected by staining for 15 enzymatic systems (Table I), 28 have been interpreted, while one esterase has been omitted. The data concerning the polymorphic systems are reported in Table IV. To clarify the complex pattern of esterases (ten activity zones of which eight are monomorphic), the heart, hepatopancreas, eye, and muscle were analyzed separately. The various loci showed distributions excluding overlaps of two distinct loci in the patterns resulting from each individual *in toto*. The 6-phosphogluconate dehydrogenase was obtained as secondary stain of the hexokinase (unpublished data).

Ophiomusium lymani

Only 13 of the 15 stains gave positive results (Table I). Among 19 detected zones, two leucine aminopeptidases were difficult to interpret and were not utilized

Table VI

Summary of genetic variation in four deep-sea species of marine animals.

Parameter	*Buccinum* sp.	*Ophioglypha bullata*	*Munidopsis hamata*	*Ophiomusium lymani*
Number of loci	29	24	29	17
Number of individuals	22	25	23	47
Genes sampled per locus	44	48.261 ± 1.221	44.857 ± 0.678	90.705 ± 1.626
Alleles per locus	1.586 ± 0.153	1.739 ± 0.157	1.379 ± 0.144	2.118 ± 0.341
Polymorphic loci	0.357	0.565	0.250	0.529
Polymorphic loci with $P < 0.95$	0.250	0.521	0.214	0.529
Frequency of heterozygotes:				
average over individuals	0.093 ± 0.008	0.122 ± 0.019	0.075 ± 0.006	0.189 ± 0.012
average over loci (obs.)	0.092 ± 0.031	0.137 ± 0.032	0.079 ± 0.033	0.191 ± 0.065
average over loci (exp.)	0.104 ± 0.034	0.224 ± 0.047	0.085 ± 0.031	0.236 ± 0.067

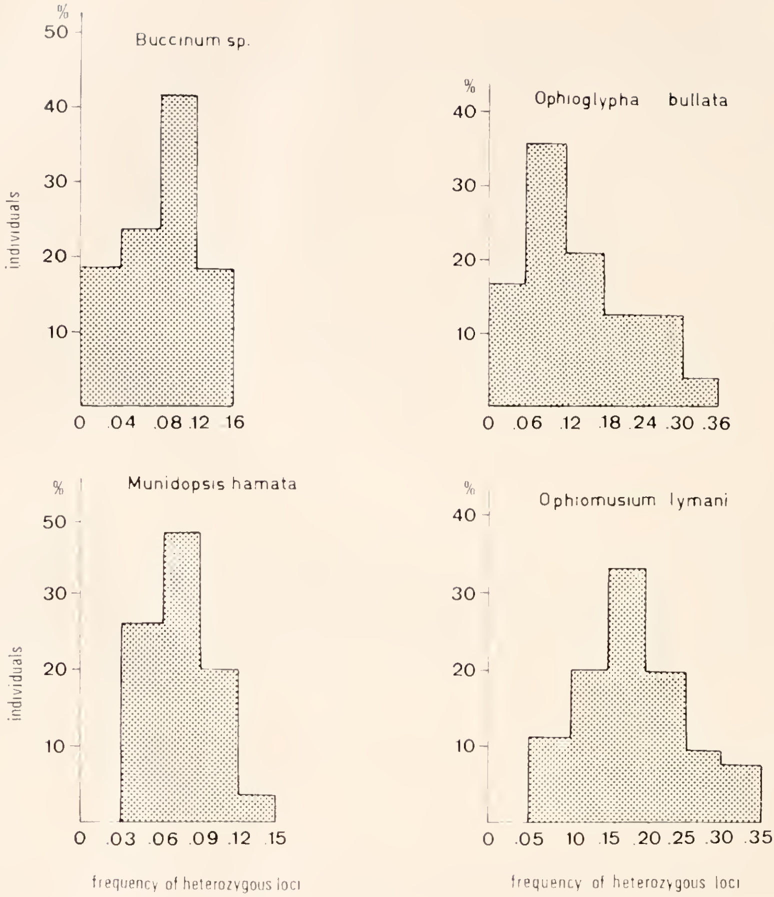

FIGURE 1. Distribution of the heterozygosities of individuals.

for the estimation of the genetic variability of this species (Table V) even though, in all probability, they were the expression of polymorphic loci as reported for a Pacific Ocean population (Ayala and Valentine, 1974).

In Figure 1 and 2 are reported the distributions of heterozygotes both for individuals and for loci. The summary data concerning the degree of genetic variability of the four species are given in Table VI.

DISCUSSION

An intermittent overflow of dense and deep waters, coming from the Norwegian Sea, flows into Rockall Trough, a depression of the Wyville- Thomson Ridge (Ellet and Roberts, 1973). Such overflow was observed and studied during the period August 24–29, 1973, by the R.R.S. CHALLENGER (Currie, Edwards and Ellet, 1974), along the western side of the Wyville-Thomson Ridge. Geological data indicate that sediments from these intermittent "overspills" can be found all along the extension of the Wyville-Thomson Ridge. This overflow is the source of the dense water in the deepest point of the Rockall Trough, and, moreover, it causes periodical variations in salinity, density and temperature (Ellet and Martin, 1973).

Our results indicate that, for the species *Munidopsis hamata* and *Buccinum* sp., the agreement of the observed distribution of genotypes with the Hardy-Weinberg is, in general, satisfied. This is not the case with several loci of the two ophiuroids, where a significant deficiency of heterozygotes appears to be the rule. In our opinion this fact should not be sufficient to invalidate the kind of genetic control postulated for the observed patterns. Artifacts due to a poor storage of the material, as well as erroneous interpretations due to between-loci hybridizations (most loci taken into account showed a single zone of reaction) can reasonably be discarded.

Moreover, previous authors, such as Doyle (1972) for an esterase locus, and Murphy, Rowe, and Haedrich (1976) for many loci, observed a significant deficiency of heterozygotes in Atlantic populations of *Ophiomusium lymani* and other ophiuroids.

Our data partially confirm the high degree of genetic variability found by other authors in deep-sea organisms. In the ophiuroid, *Ophiomusium lymani*, the esti-

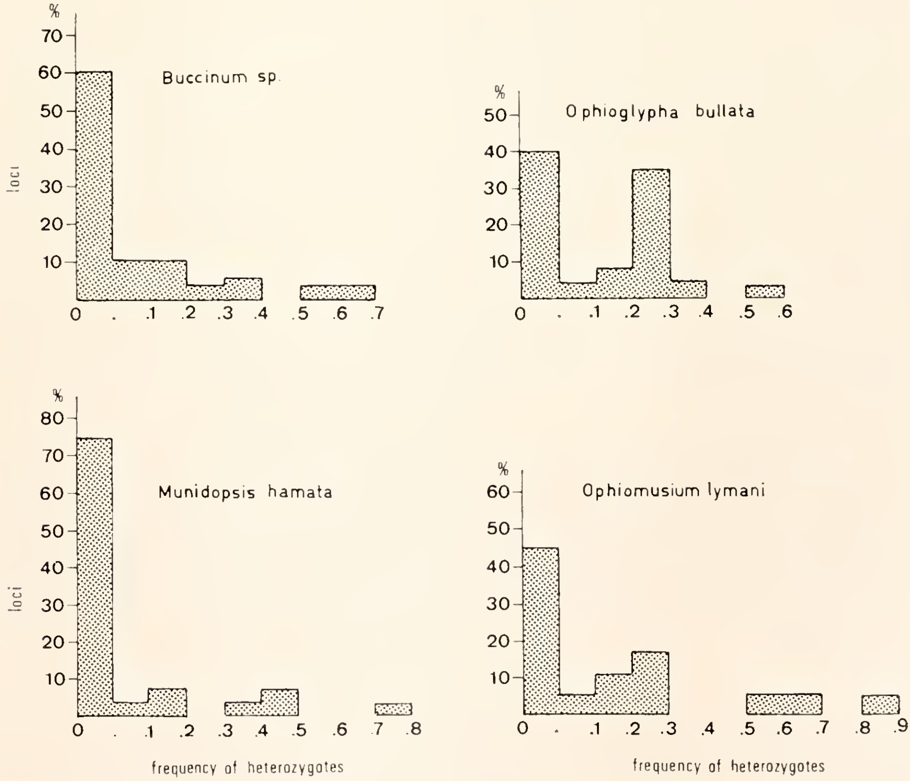

FIGURE 2. Distribution of loci relative to the proportion of heterozygous individuals at each locus.

mated frequency of the polymorphic loci, equal to 0.529, is very near to that found by Ayala in a population of the same species from the Pacific Ocean (Ayala and Valentine, 1974). In the other ophiuroid, *Ophioglypha bullata,* the frequency of polymorphic loci is 0.521. The values in the gastropod, *Buccinum* sp., and in the crustacean, *Munidopsis hamata,* 0.250 and 0.214, respectively, are clearly lower. We believe that this lower variability is not the result of a misinterpretation of the electrophoretic patterns (*i.e.,* esterases). The use of competing substrates which give differential stains, and the electrophoretic analysis of homogenates of single organ parts, supports the assumption that each apparently unvarying zone is the expression of a single locus.

The different levels of genetic variability cannot be related to the degree of environmental stability. The deep-sea organisms affected by the overspills are *Munidopsis hamata* (low variability) and *Ophiomusium lymani* (high variability), while the species *Ophioglypha bullata* (high variability) and *Buccinum* sp. (low variability) have been sampled northeast of the Wyville-Thomson Ridge in an area not affected by these intermittent overspills. This may indirectly indicate that the selective role of the environmental physical parameters is nil or very small. On the other hand, the overall genetic variability is an adequate indication of the action of selection, which can be found only in extended and periodical samplings such as to demonstrate significant variations of genic frequencies.

Ayala *et al.* (1975) hypothesized that genetic variability in deep-sea populations could be maintained by selective pressures of environmental biotic factors. With our data it is impossible either to deny or to confirm such an hypothesis. Nevertheless, from the analysis of the results, a significant consideration emerges: the different levels of variability in organisms belonging to different phyla could mean that the adaptive strategies of the species are different. It has been shown that in certain echinoderms a greater variability is found in the species from a deep-sea environment, as compared to those from shallow water (Schopf and Murphy, 1973). In the decapod, *Munidopsis hamata,* on the other hand, polymorphism is similar to that of other species of decapods coming from different environments (Hedgecock, in Valentine, 1976). The strategies of adaptation are the result of interactions between environmental factors and biological characteristics of the species, such as the life cycle, reproductive type and population size.

Our thanks are due to Dr. Dario Colombera and to the Scottish Marine Biological Association for the opportunity of taking part in the CHALLENGER's cruise. We thank Prof. Bruno Battaglia for advice and criticism received during the preparation of the manuscript. We are grateful to Drs. Roger W. Doyle, John F. Grassle and Thomas J. M. Schopf for their critical reading of the manuscript. This research was supported by a C.N.R. grant, from the Institute of Marine Biology, C.N.R., Venice, Italy.

SUMMARY

Four species of deep-sea marine invertebrates, coming from the vicinity of the Rockall Trough (Shetland-Faroe Channel), have been studied by means of gel electrophoresis. The degree of genetic polymorphism found would seem to indicate

absence of correlation with the particular environmental factors which characterize the sampled area. The results suggest that the adaptive strategies of organisms belonging to different phyla are different.

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ECOLOGY AND MORPHOLOGY OF FEEDING AND AGONISTIC BEHAVIOR IN MUDFLAT STOMATOPODS (SQUILLIDAE)

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Morphological structure co-evolves with the behavior and ecology of organisms. Food habits are influenced by the trophic apparatus, for example, and indeed differences in trophic morphology are often used by ecologists as presumptive evidence of niche differentiation by foraging strategies (*e.g.*, Schoener, 1965, 1974) or of convergent evolution in feeding methods (*e.g.*, Karr and James, 1975). Other behaviors have also co-evolved with morphology; the use of teeth, claws, chelae, etc., in agonistic encounters and displays being cases in point. In this paper data are presented relating morphology to feeding and agonistic behavior in stomatopods, including evidence that, in these animals, differences in sensory capabilities as well as differences in trophic adaptations may be associated with differences in food habits.

Stomatopods are predatory marine crustaceans occurring primarily in a variety of tropical littoral and sublittoral habitats. The chief characteristic of the group is the enlarged second thoracopod (maxilliped) which is used in prey capture and in agonistic interactions. The merus of this appendage is greatly enlarged and extends the length of the carapace or more; the terminal dactylus is also elongate and folds forward under the grooved propodus. The propodus and dactylus can be rapidly unfolded and extended to strike or seize prey or an opponent much like the raptorial prothoracic legs of a praying mantis (hence, the common name of mantis shrimp).

Stomatopods can be divided into two morphological types, the "spearers" and the "smashers", based on the structure of the raptorial appendage and the mode of feeding (Caldwell and Dingle, 1975). In the spearers the dactyl bears teeth and the propodus is usually spined (Fig. 1); these structures are used primarily for seizing and holding prey such as fish, shrimp, snapping shrimp, polychaetes and other relatively soft-bodied organisms, although some evidently take harder items such as thin-shelled pelecypods. The spearers comprise all of three of the four extant families of stomatopod, the Squillidae, Lysiosquillidae, and Bathysquillidae, and part of the fourth family, the Gonodactylidae. The remainder of the Gonodactylidae are smashers. These have a smooth dactyl and propodus (with residual dactylar teeth in some species), and the proximal end of the dactyl is swollen and functions as a hammer (Caldwell and Dingle, 1975). Prey items of smashers include primarily gastropods, pelecypods, crabs, hermit crabs and the like, although other prey can be taken if caught. Prey are captured and then demolished by repeated strikes of the raptorial appendage. Spearers occur largely in mud or sand substrates and construct their own burrows, while smashers occur in rock, coral, or rubble and occupy primarily previously existing cavities.

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Previous studies (Dingle, 1969; Dingle and Caldwell, 1969; Caldwell and Dingle, 1975, 1977) have demonstrated complex agonistic interactions both within and between species of smashers. In contrast, such behavior in spearers is much less complex, although relative levels of agonism can apparently determine outcomes in interspecific competition (Dingle and Caldwell, 1975). In this paper the relation between behavior, morphology, and habitat in five spearers is analyzed. Three species, *Oratosquilla inornata, O. nepa,* and *Cloridopsis scorpio,* are discussed extensively; and some notes on *Harpiosquilla harpax* and *O. woodmasoni* are also provided. All these species belong in the family Squillidae (Manning, 1969a, 1971).

MATERIALS AND METHODS

Most animals used in this study were collected from mudflats in the immediate vicinity of Ang Sila, Chon Buri Province,Thailand, situated on the eastern shore of the Gulf of Thailand about 90 km south of Bangkok. Burrows were located at low tide, and the stomatopods were extracted by reaching in one end of a burrow and forcing the occupant out the other (see Dingle and Caldwell, 1975 for details of sampling and a description of the area). Stomatopods were also frequently taken by fishing boats working the waters of the Gulf, and several individuals, especially of *O. nepa, O. woodmasoni,* and *H. harpax,* were acquired from fishermen.

In the laboratory the smaller species (30–100 mm), *C. scorpio* and *O. inornata,* were kept in individual containers with a blackened Erlenmeyer flask as a shelter. They were fed every 2–3 days with bits of fish or shrimp, and the water was changed after each feeding. The larger species (70–200 mm), *O. nepa* and *H. harpax,* were kept in large tanks with continuously running sea water and were also fed at 2–3 day intervals. With the exception of two small immature individuals, all specimens of *O. woodmasoni* died before reaching the laboratory.

Observations of agonistic interactions between two individuals were usually conducted between size and sex matched animals in open arenas. These had diameters approximately five times the length of the animals concerned and a sand-covered bottom. These diameters were chosen to maximize contacts between animals for assessing behavioral acts and displays and relative levels of agonism, but still to allow interacting animals to break off and remain apart. Data were voice recorded on tape during the ten minutes of each encounter. Individuals of *O. nepa* from communal tanks were kept isolated for 24 hours before observations were made. A total of 53 ten-minute interactions involving three species were recorded.

In the case of *C. scorpio* and *O. inornata,* observations of burrowing and burrow defense were conducted in large cylindrical glass containers filled to a depth of 15 cm with mud from the natural habitat. Sea water was then added to a depth of 25 cm above the mud. Once the water had cleared, a stomatopod was added and burrowing behavior recorded. Data recording was facilitated by the fact that much of the length of the completed burrow was usually visible, since most animals built against the side of the container. Approximately one day after completion of the

TABLE I

Comparison of body measurements and ratios for five species of squillids. All values are means except maximum carapace length.

Species	N	Maximum carapace length (mm)	Ratio of cornea width to eyestalk length	Ratio of antennule length to carapace length
H. harpax	5	37.6	1.52	2.12
C. scorpio	11	19.2	.70	1.36
O. inornata	11	18.8	1.33	2.13
O. nepa	11	30.0	.87	1.69
O. woodmasoni	5	25.4*	—**	2.09

* Largest museum specimen (see Methods). Specimens comparable in size to *O. nepa* were taken at Ang Sila.

** See Methods for explanation of absent *O. woodmasoni* measurement.

burrow, a second animal was introduced, and data recording was begun when it perceived the burrow entrance (indicated by an approach toward the entrance with eyes and antennules oriented toward it) and attempted entry. Data were recorded either until an eviction occurred or until five minutes had passed with no attempts by the intruder to gain entry to the burrow. A total of 32 burrow defense interactions were observed.

Morphological measurements were made with calipers on preserved specimens captured at Ang Sila, supplemented by measurements on specimens in the collection of the U. S. National Museum. The data from museum specimens are included only in the plots in Figure 3. Much of the Ang Sila collection was damaged in transit; the data in Table I are taken only from the least damaged Ang Sila specimens with the result that sample sizes are small. Differences between species, however, are still apparent. All specimens of *O. woodmasoni* were seriously damaged, so data perforce are from museum material and only dimensions least affected by preservation are included. Detailed descriptions of the various body measurements are given in Manning (1969b), and his techniques were followed in all cases.

RESULTS

Morphology and prey capture

Based on laboratory observations of prey-capture behavior, *H. harpax* is capable of catching and consuming fish without difficulty, while the other species are not. In parallel experiments eight small (40–70 mm) laterally compressed fish of the same species were introduced into each of two large aquaria: one containing seven *O. nepa* of about 180 mm long, and the other containing seven similarly sized *H. harpax*. Within a few seconds, two of the fish in the *H. harpax* tank had been captured and were being eaten, and by the end of twelve hours all had been consumed; until captured, the fish associated in a tight school. In contrast, no fish in the *O. nepa* tank were captured in the twelve hour observation period, and they did not school.

The behavior of the two species of stomatopods toward the fish was also conspicuously different. *H. harpax* stalked fish or tracked them with eyes and antennules, striking by rapid extension of propodus and dactyl when they were approximately 50–150 mm ahead of or above the stomatopod. If the strike was successful (approximately 30–50% of the time depending on the experience of the fish), the fish was clasped between dactyl and propodus and brought to the mouth where it was also held by the remaining maxillipeds while being chewed upon by the mandibles (see illustrations in Caldwell and Dingle, 1976). *O. nepa,* on the other hand, struck only when a fish was 20–30 mm in front of the head and usually only when fish actually blundered into the antennules. Two fish were captured and both easily escaped; following the escape, the stomatopod in each case swam around for several seconds sweeping the bottom of the aquarium with laterally oriented back and forth movements of the antennules, evidently seeking the prey on the bottom. On several occasions live fish were put in the home containers of individual *O. inornata* and *Cloridopsis scorpio.* In no case did an individual of either species succeed in capturing one of these fish. In fact, fish and stomatopod often shared a container for several days.

Gut analysis suggested that the diets of *Oratosquilla* spp. and *C. scorpio* consist mostly of crustaceans, annelids, and thin-shelled pelecypods (Dingle and Caldwell, 1975). It was impossible to make a quantitative assessment of dietary overlap between the species because stomatopods completely fragment food before ingestion, but the three species apparently do take qualitatively similar food items. What is also interesting is that these species all evidently take some pelcypods, which although thin shelled, still require smashing before consumption. In this respect, at least, the diets of these spearers are similar to those of smashers (Caldwell and Dingle, 1975, 1976). In contrast to their lack of success with fish, both *C. scorpio* and *O. inornata* readily captured and ate small snapping shrimp (Alpheidae) which were common in the stomatopod habitats. The diet of *C. scorpio* differs from the *Oratosquilla* species in that stomatopod remains were also found in the gut. No gut contents of *H. harpax* were identified, either because of a fish diet or because they had not fed for several hours by the time they were brought to shore by fishing boats.

The most conspicuous difference in trophic apparatus in the species studied was that occurring between the fish-catching *H. harpax* and the others. First, *H. harpax* has a propodus bearing heavy, sharp spines on the anterior margin and a dactyl with eight teeth. In contrast, *Oratosquilla* spp. and *C. scorpio* possess mostly soft, comb-like spines on the anterior margin of the propodus and five (*C. scorpio*) or six (*Oratosquilla*) teeth on the dactyl (Fig. 1). Secondly, the raptorial appendage of *H. harpax* is relatively more elongate with the folded propodus and merus extending beyond the posterior margin of the carapace (Figs. 1 and 2). This length difference is evident in Figure 3, where the "reach" of the raptorial appendage is plotted against carapace length in the five species studied. Reach is defined here as the straight line distance between the posterior edge (base) of the merus and the tip of the extended dactyl when the merus propodus angle is 135° and the propodus-dactyl angle is 90°, which seems a reasonable approximation of maximum raptorial extension in prey capture. Over individuals of all sizes, the reach of *H. harpax* is clearly greater; the points for *H. harpax* overlap those of none of the other species, while these four species extensively overlap each other. Paired comparisons of *H. harpax* with each of the other four species yielded *t* values of 11.47

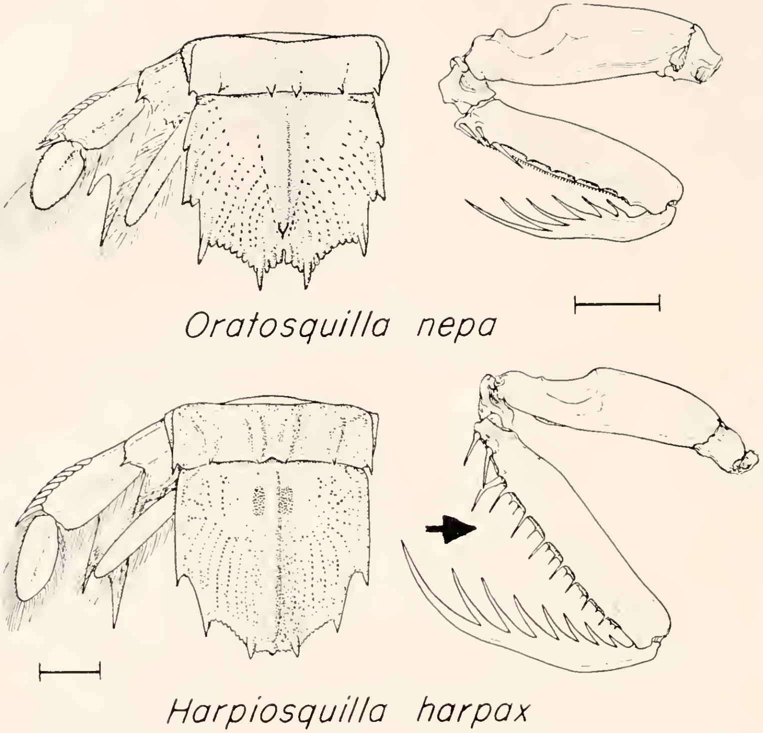

FIGURE 1. Telsons and raptorial appendages (second maxillipeds) of two spearers. Arrow indicates propodal spines of the fish eating *H. harpax*. Scale lines are 1 cm. Other species of *Oratosquilla* and *C. scorpio* closely resemble *O. nepa*.

to 18.47 which were all significant at $P < 0.001$. It seems likely that the elongated, spined, and toothed raptorial apparatus of *H. harpax* is an adaptation for capturing fish.

The raptorial appendages of *C. scorpio* and *Oratosquilla* spp. apparently differ little from each other. The various segments in all these species are of approximately the same relative length as indicated, for example, in the measurements of reach plotted in Figure 3. The similarities are also apparent in Figure 2. There are only two apparent differences. First, there are five dactylar teeth in *C. scorpio* and six in *Oratosquilla,* but this character varies between five and six both within and between species of both genera (Manning, 1969a, 1971; Tirmizi and Manning, 1968). Secondly, *C. scorpio* has a slightly heavier appendage. The mean ratio of merus width to merus length, for example, was 0.39 in *C. scorpio* (range 0.37 to 0.42, $N = 27$) and 0.33 to 0.34 in the *Oratosquillas* (range 0.31 to 0.35; $N = 6$ for *O. woodmasoni;* $N = 28$ for *O. nepa,* and $N = 29$ for *O. inornata*); *C. scorpio* was significantly different from each *Oratosquilla* at $P < 0.001$ for the Median test (Siegel, 1956). The structure of the raptorial appendages suggests that, like *O. nepa* discussed above, if any of these species were able to capture fish, they would have difficulty holding and eating them.

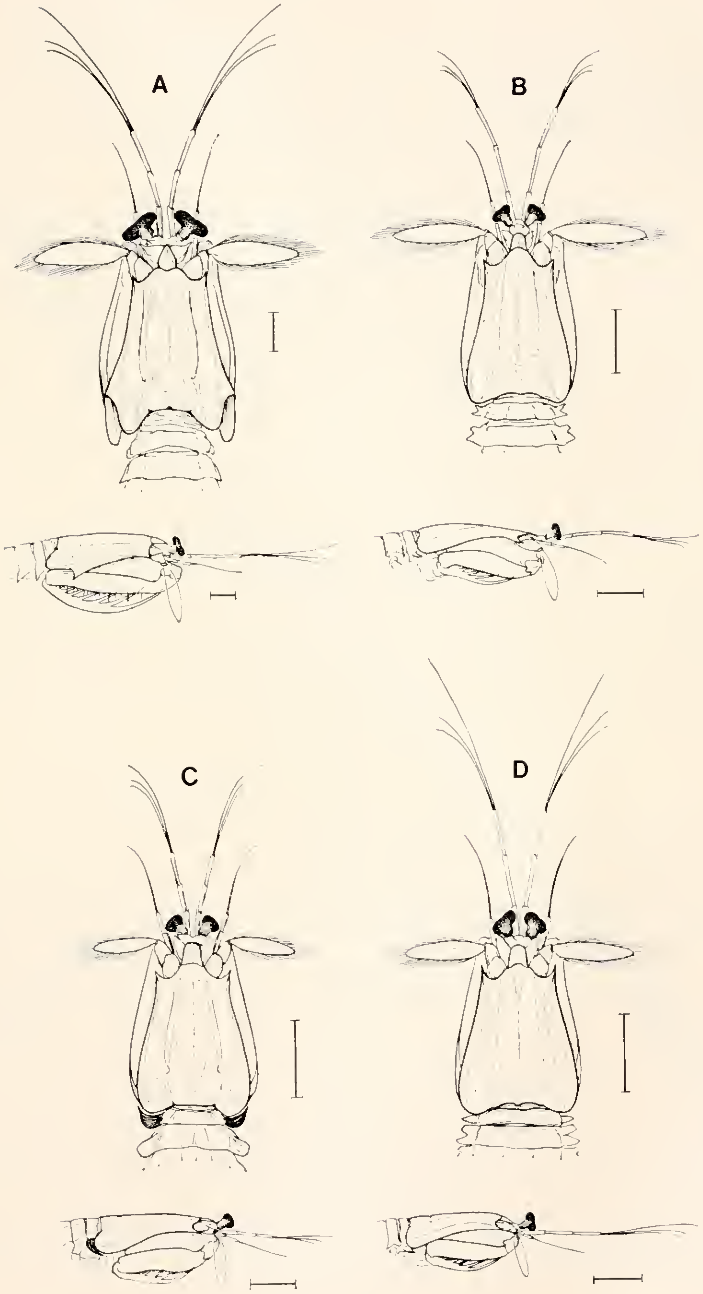

FIGURE 2. Relative proportions of cephalo-thoracic region of: A, *H. harpax;* B, *O. nepa;* C, *C. scorpio;* and D, *O. inornata.* Scale lines are 1 cm. Note differences in size of eyes and length of antennules and elongated raptorial appendage of *H. harpax.* Eyes and antennules of *O. woodmasoni* have proportions similar to those of *O. inornata.*

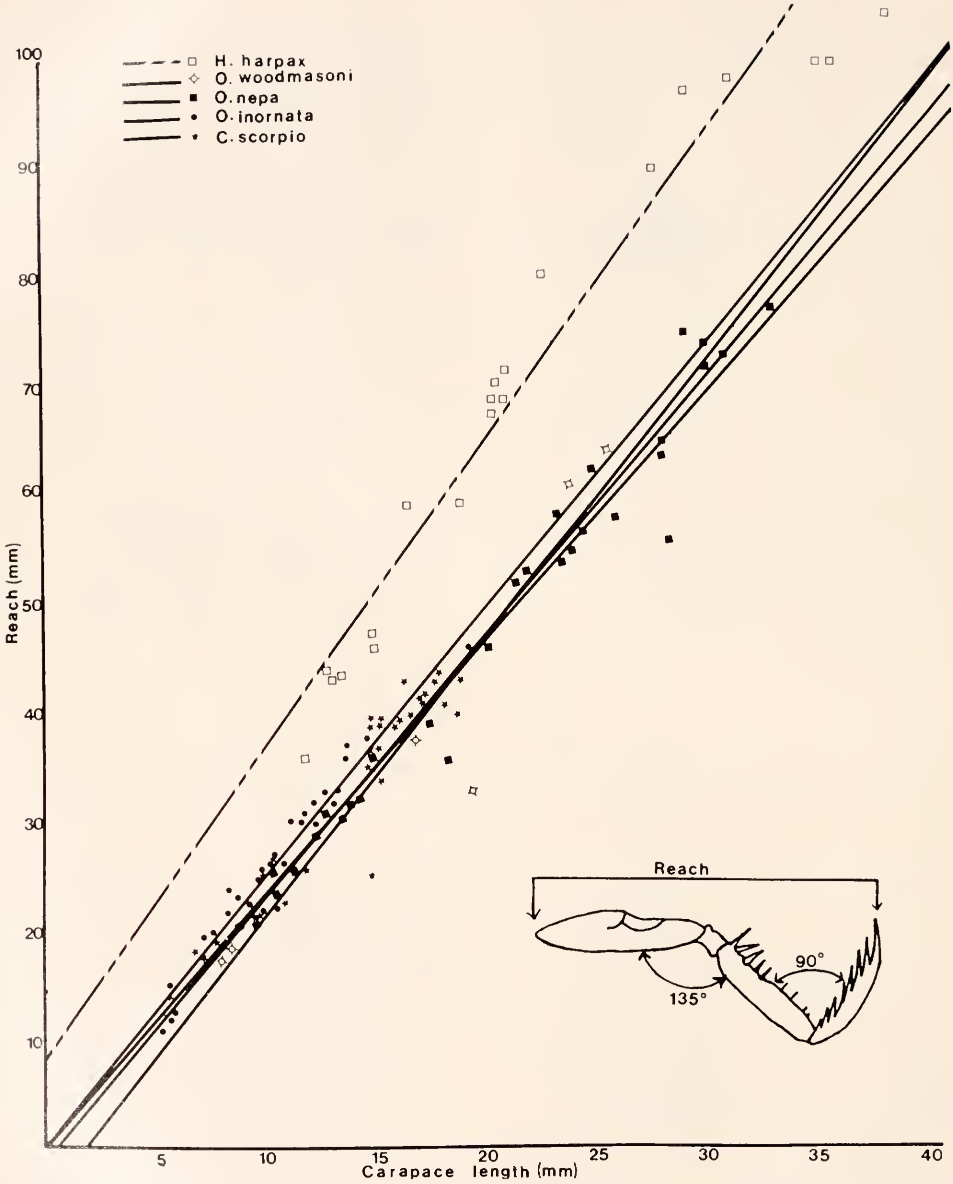

FIGURE 3. Reach (ordinate) as a function of carapace length (an indicator of body length) in five spearing stomatopods. Lines are least squares linear regressions. Note separation of *H. harpax* (a fish eater) and similarity of the other species.

The most important morphological differences among *C. scorpio* and the *Oratosquillas* with respect to prey capture may lie not in the raptorial appendages but rather in body size and the visual and chemoreceptors. These differences are shown in Table I and Figure 2. (*O. woodmasoni* is included for qualitative comparisons only, since the specimens were from a different source.) It is clear that there is, first, a division by body size into a pair of small, *C. scorpio* and *O. inornata,* and a pair of large, *O. nepa* and *O. woodmasoni,* species based on maximum recorded carapace lengths. Differences in body proportions differed little between any of the species, including *H. harpax*, as indicated by abdominal width (at the eighth adbominal segment) to carapace length ratios (range 0.95 to 1.03 over all species) or carapace width to carapace length ratios (range 0.72 to 0.78). Significant differences between species, however, do occur in eyes and antennules (bearing the primary chemical receptors), with each pair of equivalent body size having a large-eyed long-antennuled species and a small-eyed short-antennuled species (*O. woodmasoni* is large-eyed). The ranges of the ratios of cornea width to eyestalk length for the large-eyed species did not overlap those of the small-eyed, nor did the small-eyed species overlap each other ($P < 0.001$ for any paired comparison using the Median test). Similarly, the ratios of antennule length to carapace length of the long-antennuled species did not overlap those of the short-antennuled, and the latter again failed to overlap each other ($P < 0.001$, Median tests). The fish predator, *H. harpax,* has both long antennules and the largest eyes. Such differences as occur in prey taken may thus depend in part on differences in sensory structures used to detect and stalk prey.

To summarize, the fish predator *H. harpax* differs from *C. scorpio* and the *Oratosquilla* species in having a relatively longer raptorial appendage and a larger eye. These latter species differ very little in the morphology of the raptorial appendage but do differ markedly in body size or in the relative sizes of eyes and antennules.

Burrows and burrowing behavior

Burrows of all the stomatopods discussed in this paper except *Harpiosquilla harpax* were observed in the field. Of the four species whose burrows were found, *Cloridopsis scorpio* digs the most complex structure. The entrance to the burrow is a circular opening frequently covered, except for a small central opening, with a thin sheet constructed of mud, mixed with a secretion produced by the animal presumably from glands in the mouth region. The entrance leads into a smooth tube, lined with mud mixed with secretion, proceeding vertically downward for approximately 5–15 cm depending on the size of the animal. The tube then bends and proceeds horizontally and somewhat circuitously to the bottom of the entrance tube of the second entrance; the walls of this portion of the burrow were not particularly smooth and, in appropriate substrates, were lined with broken shell. Roughly one-fourth of the observed burrows possessed a third entrance so that the burrow was more or less Y-shaped, and a few had an ovoid side chamber. The diameter of the burrow tube along its entire length was only slightly larger than the diameter of the occupying stomatopod, and total length of the burrow, as measured by inter-entrance distance on the surface, was directly proportional to the size of the occupant (Dingle and Caldwell, 1975). *C. scorpio* burrows were longer than those of correspondingly sized *Oratosquilla inornata* and *O. nepa,* even without consideration of the twists and turns (Dingle and Caldwell, 1975).

The burrows of the three species of *Oratosquilla* were quite similar. Again the entrance hole was frequently covered with a cap of material made of mud and secretions produced by the animal. Beyond the entrance a smooth-walled tube, lined with mud and secretion and roughly the diameter of the animal, extended vertically downward for a few centimeters before turning 90° and proceeding horizontally to the vertical tube leading to the second entrance. In contrast to *C. scorpio* burrows, the horizontal tube was lined with smooth walls, did not twist and turn, and was greater in diameter than the occupying stomatopod. In *O. inornata* the diameter of the tube was approximately five times the diameter of the animal while in *O. nepa* and *O. woodmasoni,* it was roughly three times the diameter. Again, burrow length was proportional to length of the animal.

Laboratory observations of burrow construction by *C. scorpio* and *O. inornata* reveal that the process is similar in the two species. *C. scorpio* begins by drawing up mud with the maxillipeds, pushing it back beneath the abdomen, and then fanning with the pleopods to push the material out behind into a small mound. After a few minutes the animal is lying in a trench. It then digs forward and downward until it gathers a load of mud, and then deposits this material in the trench after turning around. At first this material removed from the incipient burrow is tamped down in the trench, but as the trench fills and the burrow lengthens, some of it is apparently mixed with secretions and pasted against the sides of the nascent entry tube. In about ten minutes the entry tube is 3–4 cm deep, and the animal then starts to dig at about a 60° angle gradually leveling off to dig horizontally. Mud is scooped up into a basket formed by the maxillipeds, the stomatopod then somersaults, and finally it brings the load to the entrance and deposits it by coming roughly half a body length out of the burrow. Any caving in of the walls is repaired immediately by plastering mud mixed with secretion over the site of the cave in. Burrows in the laboratory took 12 hours to complete, but excessive caving in as a result of loosely packed mud may have extended the time beyond that normally required in natural substrates.

O. inornata differed somewhat from *C. scorpio* in the initial stages of burrow construction in that it did not form a trench. An entry was begun by reaching directly downward with the maxillipeds, bringing up a load of mud, and dispersing it by fanning much like *C. scorpio* but less vigorously. When the tube is 3–4 cm deep, the animal dives in for the first time to obtain a load and then somersaults to bring it to the surface. From here the process duplicates that of *C. scorpio* except that the initial turn in the tube is an abrupt 90° rather than the more gradual 60° turn of *C. scorpio.* In both species, once a burrow is completed (by turning upward until the surface is reached) a cap is constructed over each entrance with a small hole in the middle often virtually invisible. The animals then spend most of their time at one entrance with only eyes and antennules projecting from the burrow and with antennules kept flat on the substrate surface.

Agonistic behavior

The behavioral acts observed in ten minute, open arena encounters between two size and sex matched stomatopods are indicated in Table II for the three species for which such data were collected. Briefly, these acts can be described as follows:

TABLE II

Number of acts of various types performed by three squillid species in unisexual paired ten minute encounters in an open arena.

Species	Acts							Antennular contact	Total	N	Mean**
	Approach	Head-up	Lunge	Strike	Chase	Avoid	Follow				
C. scorpio ♂ (I)*	9	2	9	7	2	5		17	51	6	8.5
C. scorpio ♂ (F)	1		8	1	3	37		6	56	6	9.3
C. scorpio ♀ (I)	7	3	2	6		9		22	49	7	7.0
C. scorpio ♀ (F)		1		1	7	34	1	5	49	7	7.0
O. inornata ♂ (I)	10		2	5		14		39	70	10	7.0
O. inornata ♂ (F)	2		3	2	6	83	6	11	113	10	11.3
O. inornata ♀ (I)	25		3	8		24	9	70	139	11	12.7
O. inornata ♀ (F)	3		3	5	3	126	16	13	169	11	15.4
O. nepa ♂ (I)	17			5		6		21	49	9	5.5
O. nepa ♂ (F)			1	3		27	3	29	63	9	7.0
O. nepa ♀ (I)	33		1	4		2	1	57	98	10	9.8
O. nepa ♀ (F)			1	4		68	2	52	127	10	12.7

* I = initial acts; F = following acts (see text).

** Mean = total column divided by N column (where N = number of interacting pairs) and indicates mean number of acts per 10 min interaction per interacting pair.

approach, a direct, deliberate advance toward another animal with eyes and antennules pointing forward; *head-up,* the cephalo-thorax is raised and eyes and antennules are directed forward; *lunge,* a short rapid forward movement directed to another animal; *strike,* a blow delivered to another individual by the closed dactyls; *chase,* rapid pursuit of another animal; *avoid,* rapid and abrupt swimming or turning away; *follow,* an unhurried directed movement toward an animal which is moving away; and *antennular contact,* antennular palpation of the body or "fencing" with the antennules of another animal. More detailed descriptions of most of these acts are given in Caldwell and Dingle (1975).

The data in Table II are compiled from matrices of inter-individual, two-act sequences of initial and following acts assembled for each species (several such matrices are presented in Dingle, 1969). Briefly, these matrices listed the acts which occurred as initial acts by one animal in such two-act sequences, and then indicated the number of times a specified act by the second animal followed a given initial act. The row totals thus gave the number of times each act initiated a sequence and the column totals indicated the frequency of occurrence as following acts. These totals were then arranged as the data in Table II; for each sex of each species, the number of times each act occurred as an initial and as a following act is presented. [Note that if an act was not followed by a response from the other animal, it is not included in the table. Therefore, the data here, especially those on strikes, do not exactly match those reported in Dingle and Caldwell (1975) and Caldwell and Dingle (1975) which include all acts.] Since the species discussed here spent only a small proportion of their encounter time interacting, and since their behavior consisted predominantly of the acts avoid and antennular contact, the full interaction matrices are omitted.

The predominance of these two acts, indicates that there was little agonistic behavior in open arena encounters of these three species. Antennular contact is a general response to encountering any object in the environment. It was rarely followed by an overtly aggressive response, such as a lunge or a strike, and was usually broken off when one or both animals involved avoided. Further, the most frequent response to a contact between two animals was avoidance by one and often by both. Again, response to this by the overtly aggressive act, chase, was rare in *C. scorpio* and *O. inornata* and absent altogether in *O. nepa*. If a strike, the most aggressive act, did occur, it was most frequently as an initial act in *C. scorpio* and *O. inornata;* in *C. scorpio,* strikes were almost never reciprocated with a following aggressive act and in *O. inornata* infrequently so. Strikes occurred with about equal frequency as initial and following acts in *O. nepa* but were uncommon in either case. The only possibly aggressive act which was common was approach. In these species, however, unlike the smashing gonodactylids (Dingle and Caldwell, 1969; Caldwell and Dingle, 1975), the approach is not an overtly aggressive act. Rather, it is leisurely and in no obvious way different from a directed approach to any object, aside from a potential food item, that might attract a stomatopod's attention. Similarly, following is indisinguishable from movement toward any object slowly moving away.

Thus, only three acts can clearly and unambiguously be considered agonistic: lunge, strike, and chase. Their infrequency attests to the generally peaceful behavior of the animals in these encounters. Based on the evidence available, *C. scorpio* seems to be the most aggressive species as judged by the greater proportion of these agonistic acts (Table II). The sum of all lunges, strikes, and chases in *C. scorpio* interactions was 46 acts representing 22.4% of the total of 205 acts (total column, summed) in this species. In contrast the 40 agonistic acts of *O. inornata* represent only 8.2% of the total and the 19 agonistic acts of *O. nepa* only 5.6% of the total for those species. The greatest proportion of avoids was shown by *O. inornata,* the species which overlaps *C. scorpio* most extensively in habitat (Dingle and Caldwell, 1975). Males of *C. scorpio* displayed agonistic acts more frequently than females, but there were no apparent sex differences in either of the *Oratosquillas.* Finally, head-up, a possibly agonistic act, occurred only in *C. scorpio* but did not occur with sufficient frequency to make any assessment of its role relative to agonism.

Burrow defense

Highly agonistic behavior was observed in both intra- and interspecific burrow defense interactions between individuals of *C. scorpio* and *O. inornata.* In a typical encounter an intruder, once it saw a burrow entrance, would immediately dive into the burrow, whereupon it was struck by the burrow occupant, resulting in its rapid retreat. Further attempts to enter the burrow were met in a similar manner. In both species the burrow defender occasionally came all the way out into the water column to strike. In general, *C. scorpio* was more persistant in attempting to enter burrows. This is reflected in the number of strikes delivered by the burrow occupant in the five minutes beginning with the initial attempt at entry; in six *C. scorpio* intraspecific interactions, the mean was 4.5 ± 2.4 strikes, while in seven *O. inornata* intraspecific interactions the mean was 2.14 ± 2.95 strikes. Also, in two of the *C. scorpio* cases and one *O. inornata* case an intruder gained entry, and there followed grasping with the maxillipeds and striking within the burrow itself. The act, grasp, can thus be added to the list of overtly aggressive acts in these species, although it should be noted that it occurred in burrow defense only and not in open arenas. One eviction of the burrow occupant by the intruder occurred in the six *C. scorpio* interactions, and two evictions occurred in the seven *O. inornata* interactions. Dives into the burrow, strikes, and grasps were all clearly agonistic acts.

The generally more aggressive behavior of *C. scorpio* was also demonstrated in interspecific burrow defense interactions (Dingle and Caldwell, 1975). In all eight trials in which *C. scorpio* defended against *O. inornata,* it successfully defended. In the reverse situation, *O. inornata* was evicted by *C. scorpio* in eight of eleven trials. Based on both burrow defense and open arena interactions, it can be concluded that *C. scorpio* is the most aggressive of the species studied.

Discussion

H. harpax differs from the other squillids in the overall length of the raptorial appendage, in the size of the eye, and in hunting methods. The length difference, obvious in Figure 3, clearly differentiates *H. harpax* from the other species studied, all of which were similar to each other. The greater reach and larger eye are presumably advantageous in the capture of more elusive and alert prey such as fish, for tests in the laboratory indicate that *H. harpax* is a capable fish predator.

The three *Oratosquilla* species and *Cloridopsis scorpio* all apparently feed largely on arthropods, thin-shelled pelecypods, and annelids. The similarity in raptorial appendages is striking and suggests that the evolution of the raptorial apparatus has been conservative in these genera. An example of the similarity is shown by the plot for reach (Fig. 3); dactylar tooth number is also similar. It would thus seem that resource partitioning between these species would not be on the basis of the reach or number of dactylar teeth of the trophic apparatus. This conclusion is given further support by the results of Holling, Dunbrack, and Dill (1976). They find that optimal prey size calculated from the mechanics of the trophic apparatus plotted against the cube root of body size increases linearly in a plot that includes two species of praying mantis and two stomatopods, *Pseudosquilla ciliata* and *Oratosquilla oratoria.* There is probably some partitioning on the basis of relative thickness of prey armor, since the more massive raptorial appendage of *C. scorpio* should endow this species with a more powerful strike.

In view of the qualitatively similar food items apparently taken by the *Oratosquilla* and *C. scorpio* and the marked similarity in raptorial appendages across the four species, three possible mechanisms for resource partitioning are suggested by the data. First, major differences among the species do occur in body size with two large and two small species (Table 1). Interestingly, these stomatopods do not sort out with one large and one small species per habitat as is often reported (*e.g.*, for aquatic insects, Hutchinson, 1959; Streams and Newfield, 1972). Rather, habitat sorting occurs because the small species predominate inshore where the two large species are rare (Dingle and Caldwell, 1975), and the large predominate offshore where they are regularly caught by fishermen. (Other small crustaceans were taken by the fishermen, so the small stomatopods were not simply escaping the nets.) Secondly, other observations suggest some habitat separation within each set of species. *O. inornata* on the Ang Sila mudflat tended to be more associated with rock outcrops than was *C. scorpio* (Dingle and Caldwell, 1975) and tended to occur alone, although it was relatively uncommon, on mudflats associated with mangroves. *O. woodmasoni* was far less tolerant of temperature and oxygen stress than was *O. nepa,* suggesting that it occurs on substrates less subject to fluctuations in these factors.

Thirdly, the most conspicuous differences between species within the large and small size classes of stomatopod are in the sensory structures (Table I). Although the optics of the arthropod eye are complex (*e.g.,* Horridge, 1977), larger eyes generally lead to increased visual acuity, and the fish predator *H. harpax,* indeed, has the largest eyes. The large-eyed *Oratosquillas* probably have increased visual acuity relative to the small-eyed species and increased capability of capturing more distant or more rapidly moving prey. In Crustacea, including stomatopods, the antennules are the primary appendages for chemoreception, and there is integration of inputs from these in the eyestalk ganglia (Maynard and Dingle, 1963; Maynard and Yager, 1968; Hazlett, 1971a, b). There is close coordination between these two sensory systems in prey capture, and differences could promote resource partitioning on the basis of prey type. Within body size classes and hence within habitats, differences in sensory capability could form the basis for resource partitioning as a function of prey items taken.

Differences in body proportions and in trophic apparatus have been one main focus of attention among ecologists attempting to assess resource partitioning. Indeed treatment of data with respect of these morphological variables has often reached a high level of sophistication especially with respect to birds (Schoener, 1965; Karr and James, 1975). Sensory structures have received far less attention, although differences in capability are implicit in many ecological generalizations concerning partitioning (Schoener, 1974). Our data from stomatopods suggest that greater attention to sensory differences with respect to foraging strategies may prove rewarding, especially in those cases where differences in trophic apparatus are minimal or absent.

Agonistic behavior in stomatopods has clearly co-evolved with the structure of the raptorial appendage (Caldwell and Dingle, 1975). In contrast to the species discussed here, most Gonodactylidae have a heavy hammer-like dactyl evolved for smashing hard or armored prey and generally show vigorous agonistic interaction. Only lunge, strike, chase, and in burrow defense, grasp, are unambiguously agonistic acts in these squillids. None appears to have especially evolved for agonism since all are used in prey capture; there are, in other words, no ritualized acts (Hazlett, 1972a). In contrast, the smashing gonodactylids show a highly elaborated meral display functioning agonistically as a threat, a characteristic coil used as a defense posture, and in general possess a more extensive agonistic repertoire (Dingle and Caldwell, 1969; Caldwell and Dingle, 1975).

The frequency with which agonistic acts occur also differs between these squillids and gonodactylids. The maximum mean number of acts per interacting pair in open arenas in the squillids is 15.4 per ten minute interaction for female *O. inornata* (Table II). This is less than the mean for all but one of the gonodactylids so far analyzed (Caldwell and Dingle, 1975). In the latter group of species the mean ranged from 9.4 in the least active *Gonodactylus platysoma* to 42.6 in the highly active *G. zacae*. For most of the categories in Table II, the mean is below 9.4 suggesting that these squillids are generally less active that even the most sluggish of the gonodactylids. The difference between the two families is still more conspicuous when individual acts are considered. The clearly agonistic act, strike, for example, makes up a very small proportion of acts in Table II (maximum 13.7% of the initial acts of male *C. scorpio*). In contrast, the initial acts of two typical gonodactylids, *G. bredini* and *G. spinulosus,* consist of 25.5 and 19.0% strikes, respectively (Dingle, 1968, 1972).

Two factors, prey choice and shelter availability, seem to be the major selective forces molding stomatopod agonistic behavior (Caldwell and Dingle, 1975). The closed dactyl of spearers, when used in striking, inflicts relatively less damage on another animal than the hammer-like dactyl of smashers. The squillids also live on mud bottoms and are capable of constructing their own burrows; they are thus not shelter limited. As a consequence, neither prey choice nor competition for shelter would seem to impose strong selection for well-developed agonistic behavior. However, within the group, *C. scorpio,* with the greatest investment in burrow construction and the heaviest raptorial appendage, is the most agonistic.

In the gonodactylids, the raptorial appendage has evolved to smash armored prey and has thus become an effective aggressive weapon. These species occupy pre-existing cavities in hard substrates and are apparently shelter limited. Agonistic behavior and co-evolved body armor, especially involving the telson used in the defensive coil, are therefore conspicuous features of these species. Within the group, the least shelter-limited species, *G. platysoma,* is also the least agonistic. Both within and between family comparisons suggest shelter availability as a selective agent (Caldwell and Dingle, 1975).

Parallels to the evolution of agonistic behavior in stomatopods occur in decapods. Striking and grasping are conspicuous parts of the agonistic repertoire of many species possessing a large crushing cheliped, as in crayfish (*e.g.,* Bovbjerg, 1956; Stein, 1976); whereas they are less obvious or absent when chelipeds are small as in majid crabs (Schöne, 1968; Hazlett, 1972b). Shelter-limited species such as xanthid crabs (Preston, 1973; Hazlett, 1976) and snapping shrimp (Alpheidae) (Schein, 1975) are also markedly aggressive. But in spite of these similarities, stomatopods appear to be far more conservative in the evolution of behavior and morphology. The greatest elaborations are the modifications of raptorial appendages and of telson armor in the gonodactylids accompanied by behaviors such as the meral spread and coil postures (Caldwell and Dingle, 1975, 1976). In contrast, decapod species have frequently evolved elaborate structural changes in the cheliped for use in agonistic and sexual behavior, with the snapping shrimps and fiddler crabs (*Uca*) (Crane, 1966) being outstanding examples. This paper has analyzed some of the consquences of evolutionary conservatism in the squillids; the gonodactylids will be treated in detail in future publications.

Prof. T. Piyakarnchana arranged for us to use the station at Ang Sila and, in general, smoothed our way in Thailand. Mr. S. Tameyavanish helped us locate animals and provided logistical support; Mr. P. Naiyanetr and Dr. R. B. Manning identified the species. Dr. M. Reaka and Ms. J. Dingle provided field assistance. The figures were prepared by Ms. E. Reid and J. Dingle, and the manuscript was read by Drs. R. V. Bovbjerg, F. C. James, and R. B. Manning. Supported by U. S. National Science Foundation Grants GB-37046-7 and BNS 74-22717-8.

SUMMARY

Agonistic and feeding behavior and morphological structure were studied in five stomatopod crustaceans with raptorial appendages adapted for spearing prey. Agonistic behavior was less frequent and less vigorous than in species adapted for smashing. Of the spearers, the most aggressive species, *Cloridopsis scorpio,* possessed a heavier raptorial apparatus and constructed the most complex burrow. *Harpiosquilla harpax* preyed on fish and had the longest raptorial appendages and largest eyes. The remaining species (three *Oratosquilla* spp. and *C. scorpio*) possessed similar raptorial appendages but sorted out by water depth, body size, eye size, and length of antennules. The latter two measurements suggest that differences in the structure and function of sensory systems may be important considerations in the analysis of foraging strategies and resource partitioning.

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INDUCTION OF THE WRINKLED BLASTULA FORMATION IN THE STARFISH, *ASTERINA PECTINIFERA,* BY MODIFIED DEVELOPMENTAL CONDITIONS

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The wrinkled blastula stage has been designated as a normal and definite stage in the embryonic development of a number of species belonging to the two classes of Echinodermata, Asteroidea and Holothuroidea (Newth, 1925; Dan, 1957; Hayashi, 1972). In Asteroidea more than ten species, such as *Solaster endeca* (Gemmill, 1912, 1916), *Porania pulvillus* (Gemmill, 1915), *Astropecten aranciacus* (Hörstadius, 1939), *Leptasterias hexactis* (Chia, 1966, 1968), *Mediaster aequalis* (Birkeland, Chia and Strathmann, 1971), *Certonardoa semiregularis* (Hayashi and Komatsu, 1971) and *Astropecten latespinosus* (Komatsu, 1975), have hitherto been reported as the species which pass through the wrinkled blastula stage in their normal course of development. Hayashi (1972) classified various breeding habits and larval forms of asteroids, with remarks on the wrinkled blastula, into six groups, and he suggested that the occurrence of the wrinkled blastula in some species might be due to the accumulation of a large amount of yolk during oogenesis in those species, although a close phylogenic relationship could not be detected between the species with the wrinkled blastula stage and between the species without the stage.

In the starfish, *Asterina pectinifera,* the most common species on the Japanese coast, Dan (1957) described briefly that no such phenomenon as forming a morula with many surface foldings and wrinkles could be observed. Komatsu (1972) observed the wrinkled blastula stage of this species collected on the coast of Toyama Bay, Sea of Japan, and she pointed out that the wrinkled blastula stage was inevitable in the normal course of development of this species. The recent report of Dan-Sohkawa and Satoh (1978), however, showed that most of the embryos of this species collected on the coast of Tokyo Bay, Pacific Ocean, did not form wrinkled blastulae. These seemingly contradictory reports indicate at least two possibilities on the wrinkled blastula formation of this species. One is the possibility that the wrinkled blastula formation is a rigidly planned process within the batches in some localities, and the occurrence of a wrinkled blastula would not be changed by modified environmental conditions. The alternative is that the wrinkled blastula formation of this species is rather flexible, and the formation could be modified if developmental conditions were manipulated.

The present study examines these alternative possibilities. At first, the occurrence of wrinkled blastula on two different coasts was observed in natural sea water. The results indicated that the occurrence of wrinkled blastula in this species differed from 10 to 90% among batches. Then, effects of several agents or conditions, such as osmotic pressure, salinity, temperature, pH and Ca^{++}, Mg^{++}, SO_4^{--} concentration of sea water, on the ratio of appearance of wrinkled blastula

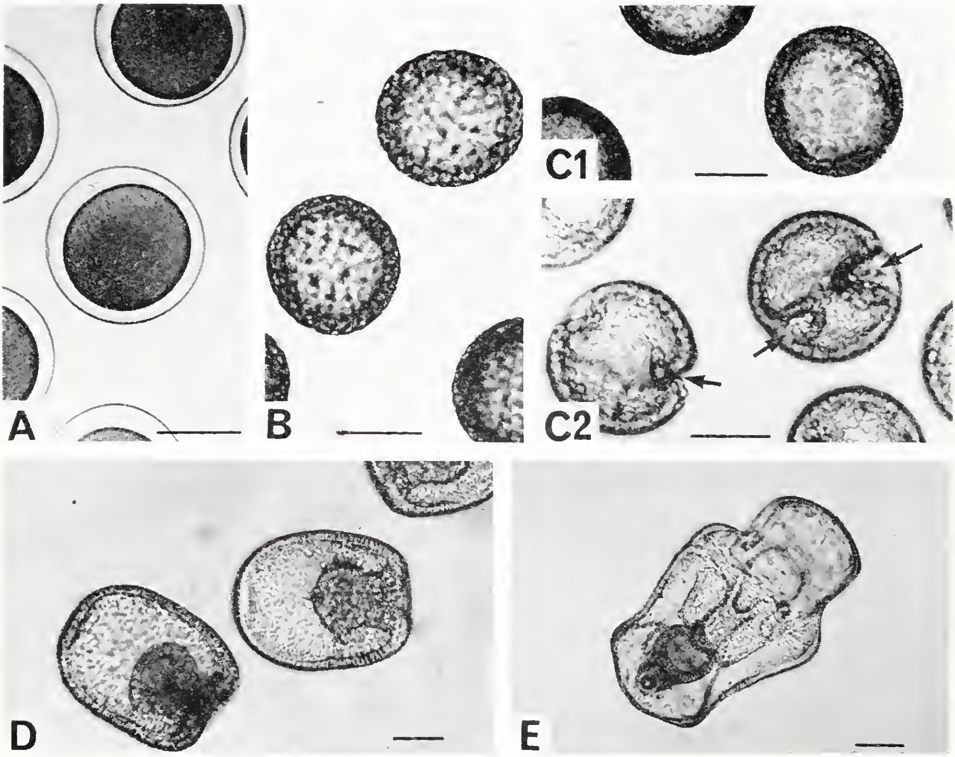

FIGURE 1. Normal embryonic development of the starfish, *Asterina pectinifera:* A, fertilized egg, 1.5 hours after insemination; B, early blastula, 5 hours after insemination; C, middle blastula, 7 hours after insemination [(1) nonwrinkled blastula with smooth surface and (2) wrinkled blastula with egression tracts (arrow)]; D, gastrula, 20 hours after insemination; and E, bipinnaria, 3 days after insemination. Mouth, oesophagus, round stomach and intestine are visible. Bar equals 100 μm.

were examined. It became clear that the wrinkled blastula formation could be increased remarkably by allowing the eggs to develop in sea water of low osmotic pressure.

MATERIALS AND METHODS

Adults of the starfish *Asterina pectinifera,* were collected on the coast of Tateyama Bay (a part of Tokyo Bay), Pacific Ocean, in June of 1977 and on the coast of Mutsu Bay, Tsugaru Channel, in September of 1977 during the presumed breeding season. By treatment with 1-methyladenine of the ovaries which were removed from the females according to Kanatani (1969), fertilizable ova were obtained. It has been shown that the wrinkled blastula formation of this species is not an artifact caused by the treatment of the eggs with 1-methyladenine (Komatsu, 1972). Dilute sperm suspension was prepared just before the insemination from mature testes removed from the males and added to the dish containing the ova.

Usually about a hundred eggs were reared in a Petri dish containing the various types of test solutions from just after the insemination. Experiments were carried out at laboratory temperature (20–22° C).

Filtered natural sea water, artificial sea water (Jamarin U, Jamarin Laboratory Co., Osaka, Japan) and Herbst's artificial sea water were used in this study. Contents of major elements in normal strength Jamarin U were as follows (g/liter): Cl = 16.947, Na = 9.311, Mg = 1.137, S = 0.774, Ca = 0.395, and K = 0.344, and its osmolarity was 0.874 osmole. Formulas for Herbst's was as follows (g/liter): $NaCl = 26.3$, $KCl = 0.7$, $MgSO_47H_2O = 11.9$, $CaCl_22H_2O = 1.5$, and $NaHCO_3 = 0.5$, and its osmolarity was about 1.030 osmole (Hosokawa and Oshima, 1971).

RESULTS

The eggs were translucent, about 170 μm in diameter (Fig. 1A). At laboratory temperature (20–22° C), they repeated cleavages every 30–40 minutes and developed into hollow blastulae about 5 hours after insemination (Fig. 1B). When the embryos developed into the wrinkled blastulae, streaks (egression tracts) appeared on the surface of the blastulae about 5.5 hours after insemination (Fig. 1C). The egression tracts gradually increased in number and size, and embryos reached the most wrinkled stage about 7 hours after insemination. Therefore, the ratio of wrinkled blastula formation was examined about 7 hours after insemination in 100–150 randomly selected eggs of each batch. The distinction between wrinkled and nonwrinkled blastulae was clear enough to exclude the possibility of misjudgement (Fig. 1C).

Five hours after the beginning of wrinkling, the surface of the blastulae resumed smoothness by the fusion of neighboring egression tracts. Eleven hours after insemination, the embryos hatched out and 30 minutes thereafter true gastrulation took place (Fig. 1D). They developed into typical bipinnaria larvae about 3 days after insemination (Fig. 1E) . Since the embryos which passed through the wrinkled blastula stage developed into typical bipinnaria larvae, the wrinkled blastula stage seems to be a completely normal process, as was reported earlier (Komatsu, 1972).

Occurrence of wrinkled blastula in natural sea water

In nine batches of eggs from Tateyama Bay, the occurrences of wrinkled blastula in the natural sea water of Tateyama Bay were 12, 14, 16, 19, 23, 40, 53, 53, and 69%, respectively. The occurrences of wrinkled blastula of ten batches from Mutsu Bay were 8, 12, 16, 17, 25, 26, 39, 41, 83, and 95%, respectively, when the eggs developed in the natural sea water of Mutsu Bay. This result indicated that the eggs of *Asterina pectinifera* pass through the wrinkled blastula stage, although the formation ratios were rather different among batches and the ratio was very low (10–25%) in more than half of batches examined.

Effects of osmotic pressure and salinity of sea water

The occurrences of wrinkled blastula in nine batches from Tateyama Bay, when the eggs developed in their natural sea water diluted with various amounts of distilled water, were examined. As clearly shown in Figure 2, the occurrences of wrinkled blastula increased in every batch in proportion to decrease in the concentration of natural sea water. For example, the batch shown by open circles, which developed about 10% wrinkled blastulae in natural sea water of normal concentration, formed more than 95% wrinkled blastulae in 0.8 times diluted sea water.

In order to examine the wrinkled blastula formation ratio in both diluted and concentrated sea water, artificial sea water, Jamarin U, was used. The result of ten batches from Tateyama Bay is shown in Figure 3. Similar to the case of the natural sea water, the occurrence of wrinkled blastula increased conspicuously in proportion to decrease in concentration of Jamarin U. In the concentrated Jamarin U (1.1–1.2 times), however, the occurrence of wrinkled blastula neither increased nor decreased. The eggs developed abnormally in 1.3 times concentrated Jamarin U.

Nearly all eggs developed into wrinkled blastulae in 0.8 times diluted natural sea water (Fig. 2) and in 0.9 times diluted Jamarin U (Fig. 3). The osmolarity of natural sea water of Tateyama Bay was about 0.980–1.020 osmole (Hosokawa and Oshima, 1971), while that of Jamarin U of normal strength was 0.874 osmole.

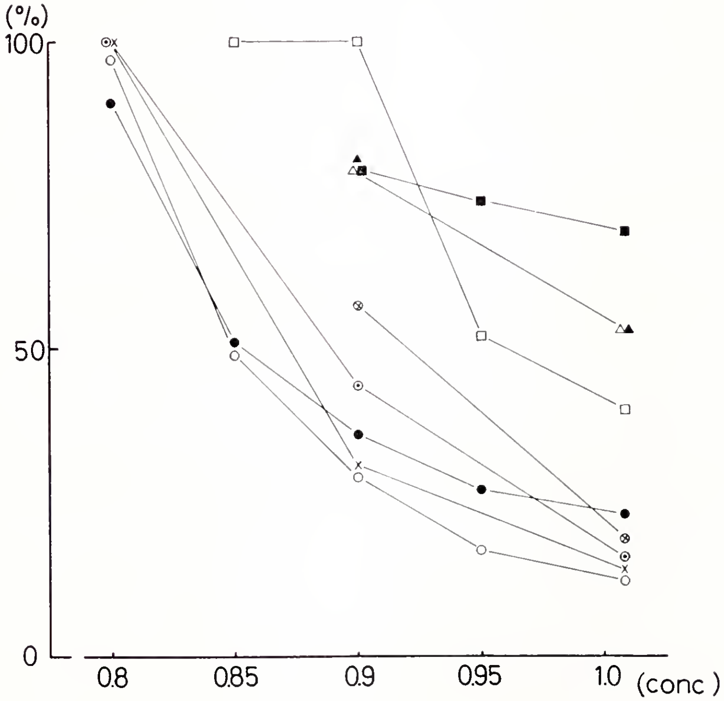

FIGURE 2. The percentage of wrinkled blastula formation in nine batches from Tateyama Bay in various concentrations of its natural sea water: the abscissa shows the ratio of wrinkled blastula formation (%); the ordinate, relative value of concentration of natural sea water diluted with distilled water (1.0 shows the normal concentration of natural sea water). The same marks tied with lines show the eggs of the same batch.

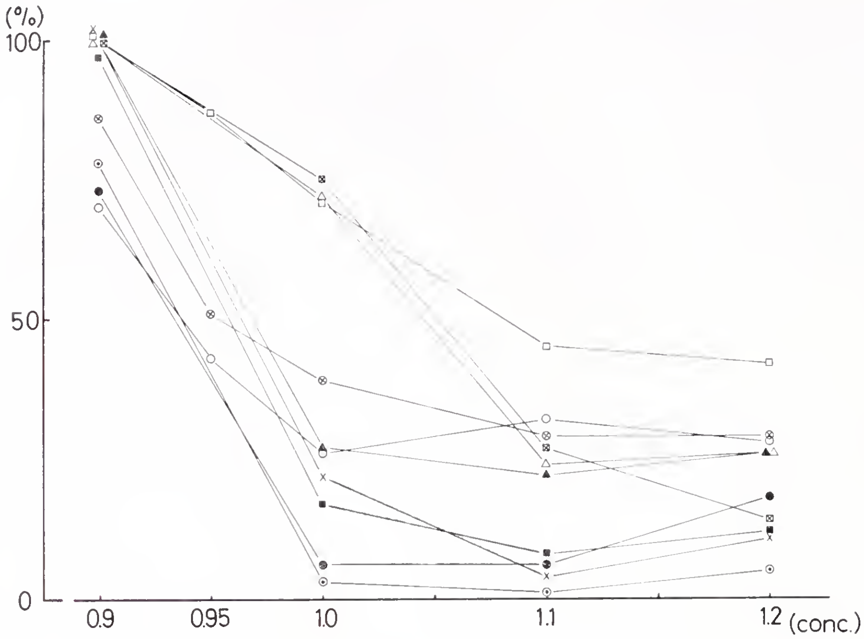

FIGURE 3. The percentage of wrinkled blastula formation in ten batches from Tateyama Bay in various concentrations of artificial sea water, Jamarin U. The same marks tied with lines show the eggs of the same batch.

Therefore, the osmolarity of 0.8 times diluted natural sea water almost corresponded to that of 0.9 times diluted Jamarin U.

The effect of dilution on the wrinkled blastula formation ratio was ascertained using Herbst's artificial sea water. The occurrences of wrinkled blastula in three batches from Mutsu Bay in Herbst's diluted with various amounts of distilled water are shown in Figure 4. The occurrence of wrinkled blastula of every batch increased in proportion to the decrease in concentration of Herbst's (Fig. 5A, B).

In order to discover whether the effect of low concentration of sea water might be due to modified osmotic pressure or due to modified salinity, the osmotic pressure of diluted sea water was maintained at normal strength by diluting the sea water with 0.75 M sucrose solution, which has been shown to be of almost the same osmotic pressure with the normal Herbst's (Hosokawa and Oshima, 1971), instead of distilled water. In all three batches, the occurrences did not increase in proportion to decrease of the saline concentration of Herbst's diluted with 0.75 M sucrose solution (Fig. 4 and 5C).

In every case the embryos which passed through the induced wrinkled blastula stage developed into normal gastrulae (Fig. 5E), although the egression tracts remained until the early gastrula stage in some cases. They became normal bipinnaria larvae.

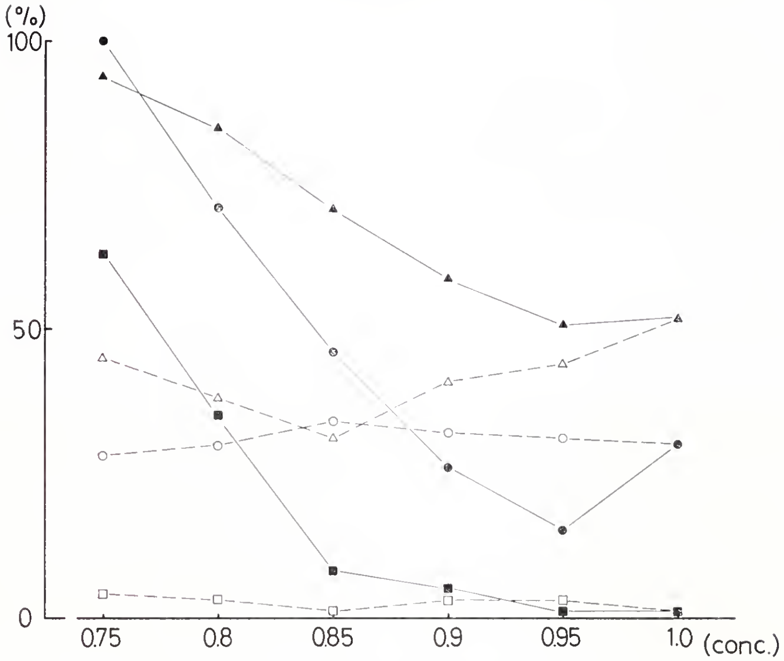

FIGURE 4. The percentage of wrinkled blastula formation in three batches from Mutsu Bay in various concentrations of Herbst's artificial sea water. The same marks, circles, triangles and squares, show the eggs of the same batch. Open marks show the ratios of wrinkled blastula formation when the Herbst's was diluted with 0.75 M sucrose solution to maintain the same osmotic pressure of the solution of different salinity, and solid marks show the ratios of wrinkled blastula formulation when the Herbst's was diluted with distilled water.

Effects of temperature

The occurrences of wrinkled blastula in two batches from Tateyama Bay and five batches from Mutsu Bay were examined at various developmental temperatures. In this experiment, all embryos were reared in Jamarin U or Herbst's of normal concentration. The occurrence of wrinkled blastula at low temperatures (13–15° C) were examined about 12 hours after insemination because of the delay of development (Fig. 6). Although the wrinkled blastula formation ratio of two batches slightly decreased at low temperatures, there was no conspicuous change in the ratio at 13–23° C. In every batch the occurrence of wrinkled blastula increased at high temperatures (25–26° C), but at such high temperatures most embryos became abnormal.

Effects of pH

The pH of normal Herbst's was adjusted by adding 30 mM tris-amino methane —0.1 N HCl, and the pH of used solution was examined again after the experiment. The occurrences of wrinkled blastula in six series of three batches from Mutsu Bay in sea water of various pH values are shown in Figure 7. Between the range

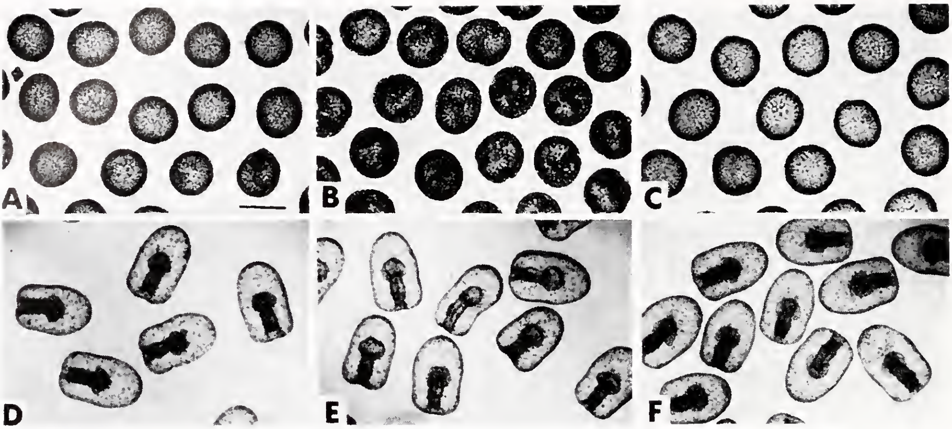

FIGURE 5. Induced wrinkled blastula formation of the eggs developed in Herbst's of low osmotic pressure. A, B, and C show the blastulae 8 hours after insemination: A, normal Herbst's; B, 0.75 times diluted Herbst's with distilled water; C, 0.75 times diluted Herbst's with 0.75 M sucrose solution. D, E, and F show the late gastrulae 24 hours after insemination: D, the late gastrulae developed from eggs shown in A; E, those from eggs shown in B; F, those from eggs shown in C. All eggs in the photographs are of one batch from Mutsu Bay. Bar equals 200 μm.

of pH 7.2–8.7, appreciable changes of the ratios were not detected. All eggs developed normally in this range of pH.

Effects of Ca^{++}, Mg^{++} and SO_4^{--} concentration

Among inorganic components of Herbst's, Ca^{++}, Mg^{++}, and SO_4^{--} were examined for their effects, since Ca^{++} and Mg^{++} have been shown to be involved in cell-to-cell adhesion, while SO_4^{--} plays some role in morphogenesis. The concentration of Ca^{++}, Mg^{++} and SO_4^{--} was changed, respectively. In this experiment Ca^{++} or Mg^{++} was exchanged for Na^+, and SO_4^{--} for Cl^-, so the total valency changed very little between the test solutions. A batch with a high wrinkled blastula formation ratio and that with a low ratio from Mutsu Bay were used. The results are summarized in Figure 8. Between the range of concentrations examined, the occurrence of wrinkled blastula did not show appreciable increase or decrease. A batch in Ca^{++}-free sea water and a batch in 1.8 times SO_4^{--} concentrated sea water increased considerably the ratio of wrinkled blastula, but the former eggs developed abnormally.

DISCUSSION

The present study suggests two results: first, the occurrence of wrinkled blastula in *Asterina pectinifera* is considerably different among batches collected both from Tateyama Bay and Mutsu Bay; and secondly, the wrinkled blastula formation can be induced by allowing the eggs to develop in sea water of low osmotic pressure.

Most reports on the wrinkled blastula in asteroids, hitherto, indicated that the

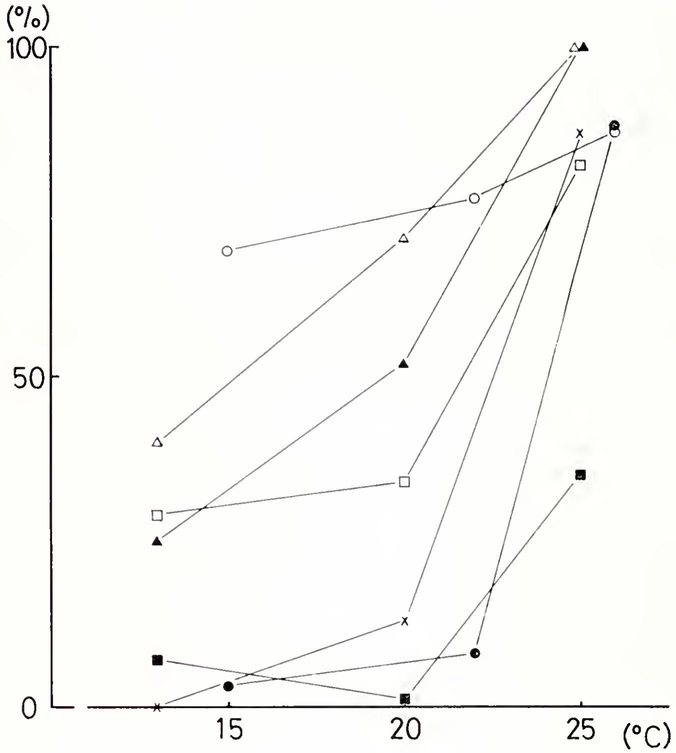

FIGURE 6. The percentage of wrinkled blastula formation in two batches from Tateyama Bay (shown by the open and solid circles) and five batches from Mutsu Bay developed at various temperatures. The same marks show the eggs of the same batch.

eggs of a certain species pass through the wrinkled blastula stage, while other species do not form the wrinkled blastula (reviewed by Hayashi, 1972); that is, occurrence of wrinkled blastula seems to have been regarded as a feature characteristic of species.

In *Asterina pectinifera,* Komatsu (1972) reported that the eggs obtained from Toyama Bay, Sea of Japan, inevitably pass through the wrinkled blastula stage. On the other hand, Dan (1957) and Dan-Sohkawa and Satoh (1978) reported that most of the embryos of this species collected on the coast of Tokyo Bay, Pacific Ocean, did not form wrinkled blastulae. However, these reports did not present any actual percentage of wrinkled blastula formation. The present results indicate that the occurrence of wrinkled blastula was very low (10–25%) in more than half of the batches examined. Since many eggs of the species from both Tateyama Bay and Mutsu Bay developed, without passing through the wrinkled blastula stage, into normal bipinnaria larvae, the wrinkled blastula stage is not an integral part of this starfish's development. In the European species, *Asterina gibbosa,* Newth (1925) noted the occurrence of a wrinkled blastula stage, although MacBride (1896) did not mention wrinkling in the same species. It is worth

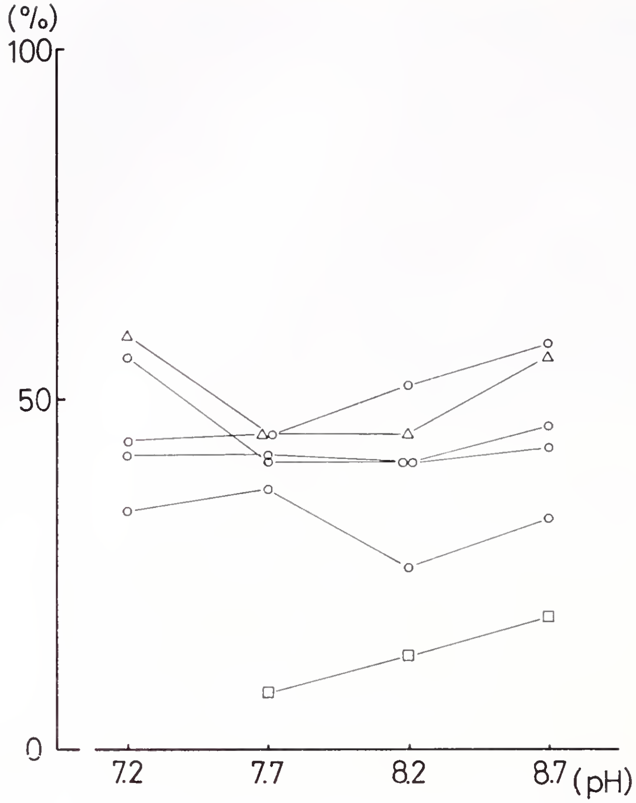

FIGURE 7. The percentage of wrinkled blastula formation in six series experiments of three batches from Mutsu Bay in sea water of various pH values. The same marks show the eggs of the same batch.

mentioning the possibility that in *A. gibbosa,* some eggs pass through the wrinkled blastula stage while others do not, similar to the case of *A. pectinifera* which has been clarified in the present study. These species are of special significance for analyzing the nature of the wrinkled blastula.

This study seems to be the first experimental analysis of the phenomenon of the starfish's wrinkled blastula. The results of the present study show clearly that sea water of low osmotic pressure increases the wrinkled blastula formation ratio, but modified salinity, temperature, pH, and Ca^{++}, Mg^{++}, SO_4^{--} concentrations give no effect on wrinkled blastula formation. Whether or not such low osmotic sea water pressures would happen naturally is not certain. The induced wrinkled blastula continued to develop and became normal bipinnaria larva in such low osmotic sea water. The mechanism involved in the induction of wrinkled blastula formation, as well as the wrinkled blastula formation in normal developmental conditions, is still obscure. In any event, much more information is needed for an understanding of the wrinkled blastula of starfish.

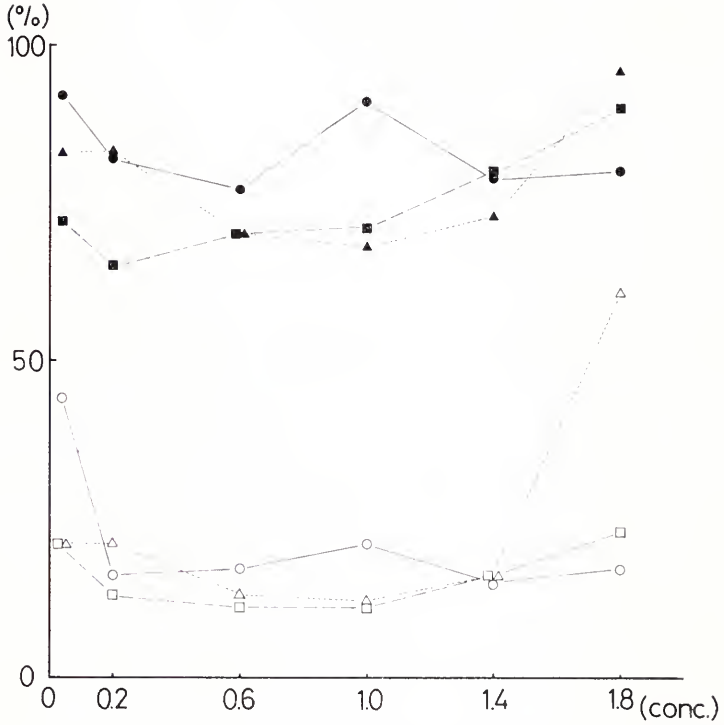

FIGURE 8. The percentage of wrinkled blastula formation in two batches from Mutsu Bay developed in sea water of various Ca^{++}, Mg^{++}, and SO_4^{--} concentrations. Ordinate shows the relative value of each ion concentration (1.0 shows the normal concentration of each ion in Herbst's artificial sea water). Circles show the ratios in various concentrations of Ca^{++}; squares, Mg^{++}; triangles, SO_4^{--}. Open marks show the first batch; and solid marks, the second.

We wish to thank Dr. S. Nemoto for providing facilities for this work at Teteyama Marine Biological Laboratory. Our gratitude is also due to Dr. M. Dan-Sohkawa and Dr. S. Hirai for their kind supply of the materials at Mutsu Bay. We also wish to thank Prof. M. Yoneda for his critical reading of the manuscript.

SUMMARY

1. The occurrence of the wrinkled blastula in the starfish, *Asterina pectinifera,* was examined. The ratios were different, from 10 to 90%, among batches. The wrinkled blastula stage is not always an inevitable process during the embryonic development of this species.

2. Low osmotic sea water (both natural and artificial sea water) remarkably increased the ratio of wrinkled blastula, while modified salinity, temperature, pH and Ca^{++}, Mg^{++}, SO_4^{--} concentrations of sea water gave no effect on the ratio. The induced wrinkled blastula developed into normal bipinnaria larva.

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A PRELIMINARY STUDY OF MECHANORECEPTORS WITHIN THE ANTERIOR BYSSUS RETRACTOR MUSCLE OF *MYTILUS EDULIS* L.

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Few studies have been performed on the anatomy and neurophysiological behavior of sensory receptors in the muscles of molluscs. Turner and Nevius (1951) were the first to report on mechanoreceptor activity in the foot of *Ariolimax*, a gastropod. Mechanoreceptor activity has been recorded in the cephalopod (Gray, 1960) and gastropod mantle (Laverack and Bailey, 1963) and the gastropod buccal mass (Laverack, 1970; Kater and Rowell, 1973). Mellon (1969) has studied the possible role of stretch receptors in the swimming behavior of the scallop.

The present work has been carried out on the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* L. This smooth muscle responds to direct current stimulation with a tonic contraction, which continues long after stimulation has ceased, while it gives a phasic contraction in response to stimulation with alternating current or repetitive pulses of direct current (Winton, 1937; Jewell, 1959).

Even though much work has been done, comprehending the underlying physiological mechanisms controlling phasic and tonic contraction in molluscan smooth muscle still remains a challenge. An approach that may elucidate these mechanisms is to record proprioceptor responses from the ABRM in response to mechanical stimuli. Such an approach can provide an index of the type of information the animal receives about the state of these muscles; further analysis might reveal the contribution of this sensory information in the regulation of the contractile states mentioned previously. In this report certain electrophysiological characteristics of mechanoreceptors found in the ABRM are described.

MATERIALS AND METHODS

Specimens of *Mytilus edulis* L. were collected at Ram Island, Connecticut, and stored in tanks of flowing sea water at 4° C. The collected mussels were 2–6 cm long. Experiments took place within six hours after each collection.

In order to record electrophysiological activity from mechanoreceptors within the ABRM of an intact animal, a special preparation was developed. The animals were mounted with the left valve up on a piece of rectangular wood (oak) cemented to the middle of the bottom of an open plastic box. Mounting was accomplished by securing the lower valve rigidly to the wood by screws passing through the holes drilled in it. The preparation was immersed in natural sea water at 6–10° C.

After mounting, a large hole was cut in the left (top) valve; this was followed by partial removal of the mantle, exposing part of the left ABRM, pedal ganglion,

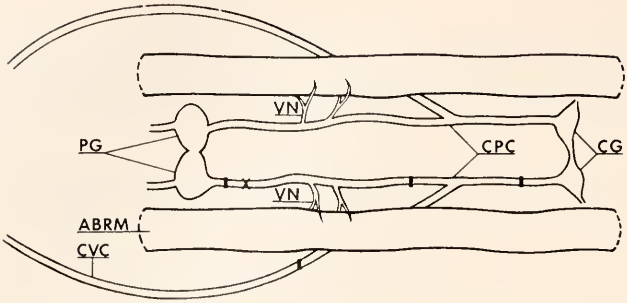


FIGURE 1. Isolation of the ABRM and its associated nerve, the cerebropedal connective nerve (CPC), from the nervous system. Bars represent the points at which the nerve was severed; and an X shows the point of hooking by an extracellular electrode.

cerebropedal connective (CPC) nerve, part of the foot, part of the cerebrovisceral connective nerve, and the visceral nerves.

At this point it was important to assess that the animals were in good condition. The criteria were that the mantle should not tear away from the shell upon light teasing of the tissue, and the foot, on being mechanically stimulated, should be sharply retracted. If these conditions were not met the animal was eliminated from the study. Any mussels with visible bacterial, fungal or nematode infestations were also not used.

By severing selected nerves at indentifiable points in the nervous system, the left ABRM and its innervating visceral nerves arising from the CPC were isolated from the rest of the nervous system (see Fig. 1). According to Twarog's (1960a) study, the innervation of the ABRMs comes entirely from the cerebral-pedal connectives, between the pedal ganglia and the branching of the cerebrovisceral connectives through the visceral nerves.

To measure muscle tension, a nonelastic cord was attached to a crop of byssus threads by surgical silk at one-half the length of the byssus threads. The other end of the nonelastic cord was attached to a force/displacement transducer.

Recording from the CPC nerve was accomplished by hooking the nerve with a platinum hook electrode insulated down to the hooked tip with GC Electronics Liquid-Tape. Drying of the CPC nerve was prevented by enveloping the hooked CPC nerve recording site with vasoline diluted to a workable consistency with parafin oil. The reference electrode, constructed the same way as the recording electrode except the bare tip was left without a hook, was placed near the byssal gland.

Mechanical stimulation was produced from a modified loud-speaker, driven by a signal generator with various amplitudes and frequencies. A plastic post from the middle of the loud-speaker impinged at right angles upon the nonelastic cord at one-half its length. Powdered molybdenum, a lubricant, was added to the point of contact between the probe of the modified loud-speaker and the nonelastic cord. The above method allowed direct measurement of tension on the ABRM. The signal generator which drove the loud-speaker also produced a synchronized trigger pulse which was recorded along with a muscle force on an FM tape recorder. Figure 2 illustrates the stimulating system.

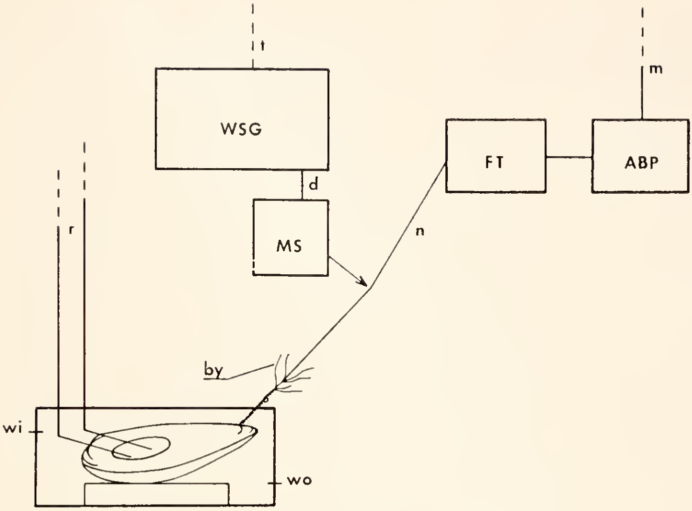


FIGURE 2. Schematic drawing of stimulating system. Abbreviations are: ABP, author built preamplifier; by, byssus threads; d, modified speaker's drive; FT, force transducer; MS, modified speaker; n, nonelastic cord; r, recording electrodes; wi, water in; wo, water out; and WSG, wavetek signal generator.

All electrophysiological recordings were amplified and then stored on a tape recorder. Conditioned data were then fed into a computer of average transients (signal averager) which produced synchronized stimulus histograms, or viewed directly on a strip-chart recorder or a storage oscilloscope.

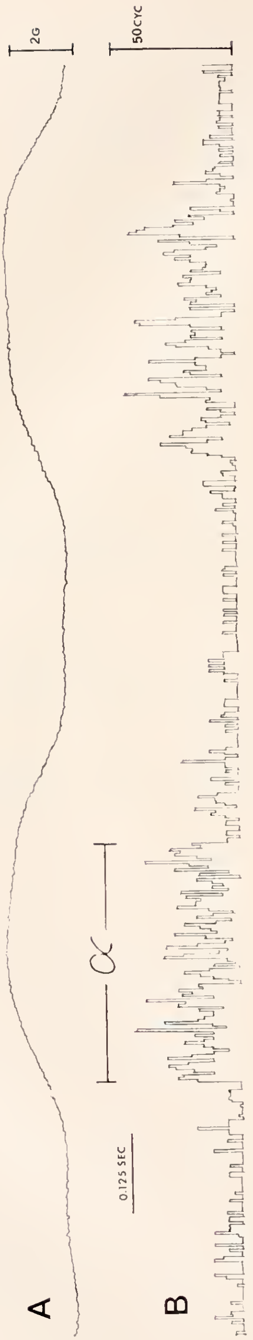
Measurement of a mussel's overall length was used for classification purposes instead of the ABRM's length because the preparation did not permit direct measurement of the ABRM's length.

RESULTS

Mechanical stimulation of an ABRM, neurally isolated except for its innervating visceral nerves and a portion of the CPC nerve, caused a multiunit discharge of impulses in the isolated CPC nerve, recorded by the platinum electrodes.

In order to determine the best stimulating frequency for further experimentation, the tension on the ABRM was varied sinusoidally around a mean value which was greater than the peak-to-peak (ppk) value of the sinusoid. Mussels of the same size were studied. Simultaneous, averaged records of tension and electrical activity of the mechanoreceptors over fifty cycles of each frequency were compiled by a signal averager. It was found that a 1.0-Hz mechanical stimulus produced the largest response of the mechanoreceptors. A 2.0-Hz mechanical stimulus is too fast for the sensory system to respond to, while a 0.1-Hz mechanical stimulus is too slow, not yielding a response over the background noise of the CPC nerve's spontaneous discharges. The activity of the mechanoreceptors due to a 0.5-Hz mechanical stimulus was not as large as the activity due to a 1.0-Hz stimulus.

Figure 3 shows a response of the mechanoreceptors to a 1.0-Hz sine wave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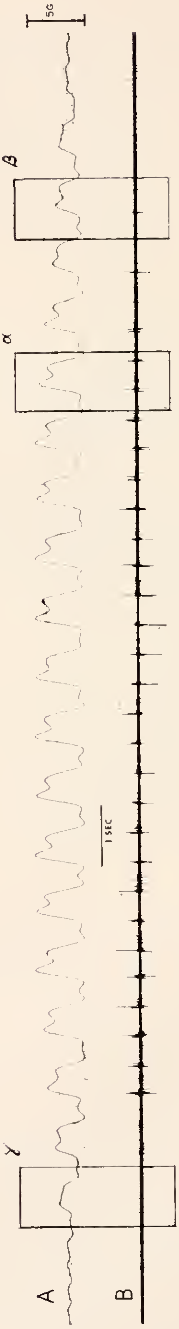
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mechanical stimulus which provides a ppk tension on the ABRM of 2 grams and a resting tension without external disturbances of 1.75 grams. It is seen from the record of the synchronized stimulus histogram that the mechanoreceptors produce firings between 1.75–2.75 grams (α of Fig. 3). These mechanoreceptors appear to be sensing stretch activity.

A response of the mechanoreceptors to a 1.0-Hz square wave stimulus is illustrated in Figure 4. The overshoot that appears on the square wave is probably due to the viscoelastic properties of the ABRM interacting with the stimulating loud speaker. The tension of the ABRM starts at rest at 6 grams, increases slowly through a few cycles of stimuli to a ppk tension of 4 grams and then finally decreases to the resting tension again. It is shown that some mechanoreceptors fire between 4.25–4.50 grams (*e.g.*, α of Fig. 4) with rising tension and others fire between 6.75–7.0 grams (*e.g.*, α of Fig. 4) falling tension. As can be seen in Figure 4, a threshold for firing can be shown as the amplitudes and rate of tension increases or decreases; as firing is absent (β and γ of Fig. 4) at lower levels of tension.

In all, three preparations each of four different mussel sizes were investigated. In the preparations, the resting tensions, the ppk values of the sine and square wave stimuli were varied but the stimulating frequency remained at 1.0-Hz. It was found that the mechanoreceptors' firings were repeatable, as shown in Figure 4. Threshold levels of tension for mechanoreceptors' firings were found to vary in position and number in relation to the animal's size.

The mechanoreceptors' responses to loading are distinct from those responses to unloading. As shown in Figure 5, the receptors responding to the rising phase (+) have spikes of large amplitude, while those responding to the falling phase (−) have lower amplitude spikes. These responses are repeatable. This evidence suggests that there are two functionally independent sets of receptors in the ABRMs which respond either to stretch or the release of tension.

Discussion

Mechanically sensitive receptor units have been found in the ABRM of *Mytilus edulis* L. The receptors responsible for the electrophysiological sensory activity within the ABRM may be the same putative receptors described morphologically by Gilloteaux (1971, 1972) as "neuro-muscular associations." Gilloteaux (1972) states that each neuro-muscular association consists of a nerve branch forming the nerve ending, generally in the form of a large spiral rolled around the modified smooth muscle fiber. Similar neuro-muscular structures were observed morphologically by Coujard (1950) in the Auerbach intestinal plexus of the rat and of the African lung-fish, *Protopterus*. These neuromuscular associations were interpreted (Gilloteaux, 1971, 1972) as interoceptors. Analogous neuromuscular associations have been observed using methylene blue staining techniques under the

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Figure 3. [On left.] Mechanoreceptors' responses to sine wave stimuli: (A) mechanical stimulus; (B) synchronized histogram. The mussel size is 4.3 cm.

Figure 4. [On right.] Mechanoreceptors' activity: (A) mechanical stimulus; (B) sensory activity of the mechanoreceptors due to square wave stimuli. The mussel length is 4.3 cm.

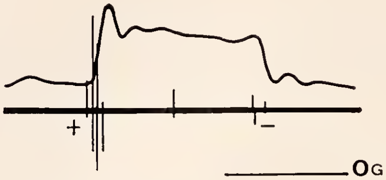

FIGURE 5. Independence of mechanoreceptors' firing through rising and falling tension. Rising tension receptors indicated by a positive sign; fire with larger amplitude than falling tension receptors indicated by a negative sign. The total sweep is one sec, and the stimulus is one cycle per second with a resting tension of 6 grams and a ppk value of 4 grams.

light microscope by LaCourse (1977) in the ABRM of *Mytilus*. In addition to Gilloteaux's description of the association, LaCourse has observed a bipolar cell, just before the nerve of the neuromuscular association terminates in the muscle fiber. LaCourse (1977) speculated that the bipolar cell is probably the soma of a sensory neuron, and the total bipolar cell with the neuromuscular structure is a mechanoreceptor. Similar bipolar cells have been observed embedded in connective tissue around the shafts of thick apodemes of the tailspine muscle of *Limulus polyphemus* (Eagles and Hartman, 1975). Eagles and Hartman speculated that these bipolar cells are tension receptors.

It is suggested by the present authors that the ABRM contains mechanoreceptors. These sensory receptors shown in the present electrophysiological study may arise from the morphologically observed neuromuscular associations described above.

From the results of the present investigation and evidence cited in the previous discussion, a speculative model for phasic contraction of the ABRM of *Mytilus* is offered. Figure 6 is a schematic representation of the model. The contractor innervation is assumed to arise in or beyond the pedal ganglion (from morphological observation of nerve tracts connecting the pedal nerve to the cerebropedal connective nerve, LaCourse, 1977), pass anteriorly along the cerebropedal connective nerves, through the visceral nerves to the middle third of the ABRM. There it branches into a fine nerve plexus covering the muscle fibers. When the motor nerves are stimulated, the plexus releases acetylcholine which acts directly on the muscle membrane to cause depolarization and contraction (Twarog, 1954, 1960b, 1967b). In turn, the mechanoreceptors located on the muscle fibers elicit potentials that are proportional to the stretch (rising or falling) on the ABRM. The elicited receptor firing is fed back to the pedal ganglion for further motor control.

The relaxor nerve fibers follow the same pathway to the muscle as the contractor innervation. When muscle relaxation is ordered, relaxing nerves are stimulated. Five-hydroxytryptamine is released from these nerves, causing relaxation of the muscle fibers (Twarog, 1954, 1960b, 1967a; Northrop, 1964). The effect of 5-hydroxytryptamine on the mechanoreceptors is not known at this time.

In essence, it is hypothesized that the mechanoreceptors are part of a sensory feedback system which supplies information to the pedal ganglion for motor control of the ABRMs. This investigation has raised interesting questions about the details of the ABRMs motor control system. It is evident that much work has yet

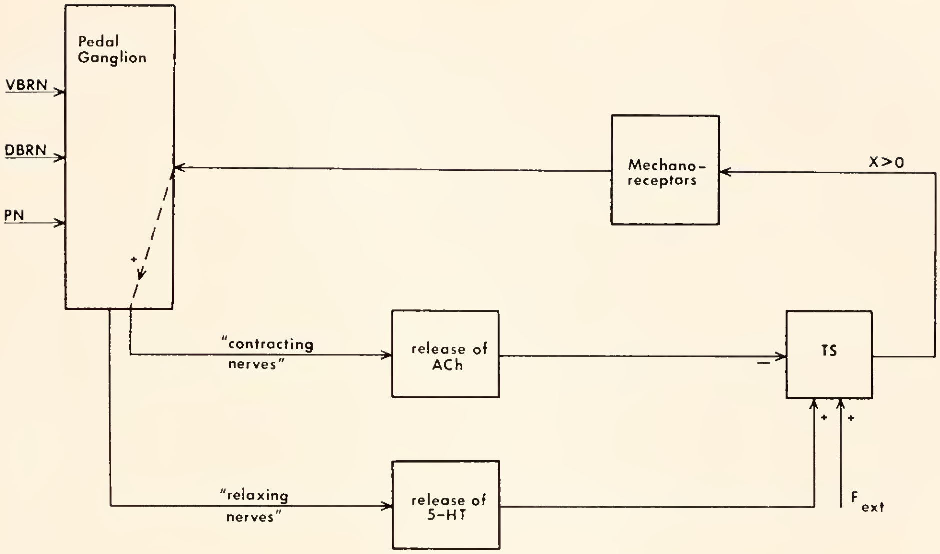

FIGURE 6. Proposed model for phasic control of ABRM. Abbreviations are: TS, nonlinear transfer function, relating muscle length to tension; F, external load; $X > 0$ is muscle lengthening; pluses on TS shows parametric control, VBRN, ventral byssus retractor nerve; DBRN, dorsal byssus retractor nerve; and, PN, pedal nerve.

to be done to clarify the closed-loop neurophysiological mechanism involved, and its relation to the so-called catch state of the ABRM.

This research was supported in part by Grant 0505-35-184 from the University of Connecticut Research Foundation. We would also like to thank Dr. Sung Y. Feng, Director, Marine Research Laboratory, University of Connecticut for his helpful advice and the use of MRL facilities throughout this study.

SUMMARY

1. Electrophysiological studies of mechanoreceptors within the ABRM of the mussel, *Mytilus edulis* have revealed two types of tension receptors. Multiunit recordings were made from an isolated nerve-muscle preparation during stretch with platinum hook electrodes. The responses observed were: type one, showing receptor firing during the rising phase of applied tension and type two, showing receptor firing during the falling phase. Based on electrical waveforms of multiunit recordings, both types of receptors seem to fire independently of each other, and can be reliably excited by the stimulus.

2. These mechanoreceptors are suggested to be part of a system which supplies information to the pedal ganglion for motor control of the ABRM.

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STRENGTH AND DESIGN OF SHELLS OF THE TWO ECOLOGICALLY DISTINCT BARNACLES, *BALANUS BALANUS* AND *SEMIBALANUS* (*BALANUS*) *BALANOIDES* (CIRRIPEDIA)

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The principal function of the shell of the barnacle is to protect the animal from mechanical damage and predation. It has other functions. Some are physiological: to provide thermal insulation, and to prevent water flux inward from hypotonic estuarine or rain water, or outward when the animals are exposed to air. Some are anatomical: to support the animal's body and provide sites of muscle attachment so that the animal can feed and perform other activities effectively. These latter activities could be carried out with fairly delicate structures, however, so the rather thick exoskeletons of barnacles are presumably designed mainly to resist mechanical damage.

Different species of barnacles in the same part of the world may have characteristically different shells, so it is relevant to enquire whether these different shells have different mechanical properties. This paper shows that the shells of the two barnacles, *Balanus balanus* (L.) and *Semibalanus balanoides* (L.) (= *Balanus balanoides*) have remarkably different strengths. This difference is most probably due to differences in general architecture of the shells rather than to microstructure and mechanical properties of the shell material. The architectural and mechanical differences between these two species are best understood in the context of their ecology and life histories.

Barnes, Read, and Topinka (1970) reported on the strengths of the shells of nine species of barnacles. They concluded that shell strength was a function of species, of shell size and of the geometry of the junctions between adjacent wall plates. They found an apparent relationship between shell strength and their ideas of the mechanical demands from waves and wave-washed debris in the habitats typical of the species they discussed.

Materials and Methods

Mechanical testing

Specimens of *Semibalanus balanoides* from the mid to low intertidal and *Balanus balanus* from the subtidal were collected from the waters of the Clyde Estuary, Scotland, and kept in tanks of chilled sea water at the University of York. The barnacles were kept on their various natural substrata: the shells of the winkle, *Littorina littorea*; the scallop, *Chlamys* sp.; the mussel, *Mytilus edulis*; the clam *Venerupis pullastra;* and on rock. The tests were made with an Instron table model testing machine. The shell or rock to which the specimen was attached was rigidly fixed so that the opercular opening was horizontal. The shell was loaded

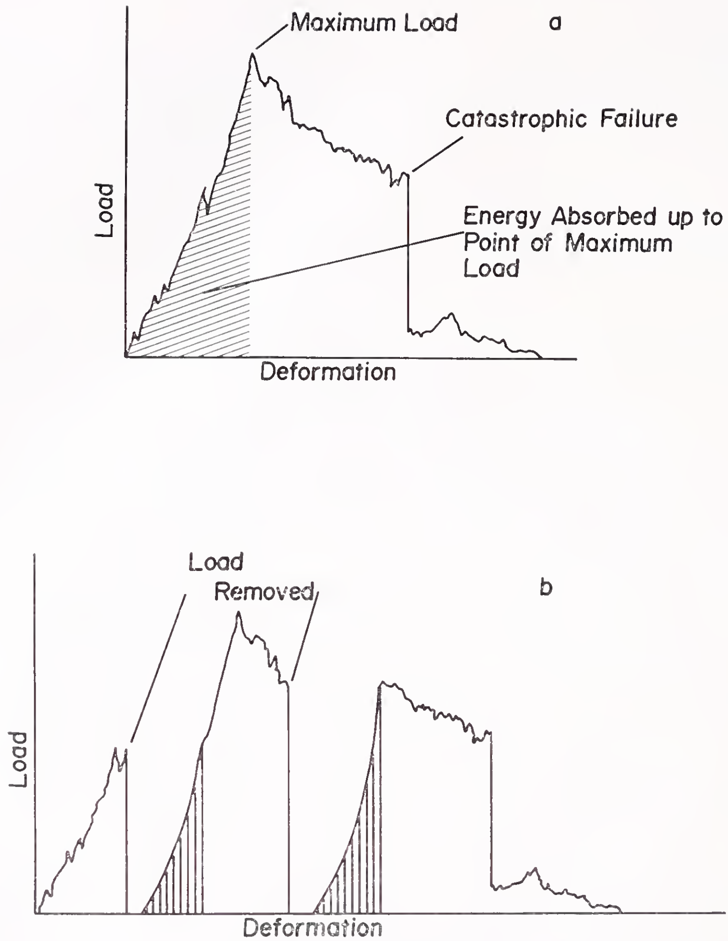


FIGURE 1. Idealized load-deformation curve for a barnacle shell strength test on the Instron. Part a shows a test trace run without pausing or as reconstructed from a test with pauses. The diagonal shading indicates the work done on the specimen up to the point of maximum loading which is the same as the energy absorbed by the specimen. Part b shows the trace obtained when there were pauses in the test (the first two vertical drops in the trace) during which the load was removed. On resumption of the test, the area under the curve up to the last value recorded before the pause (vertical shading) was ignored.

on the rim of the opercular opening by the flat end of a stainless steel cylinder on the moving head of the Instron. The shells chosen for loading were regular in shape and were hardly, if at all, buttressed by the shells of neighbors. When the base of a shell did touch another animal's shell, it was tested only if we considered that the amount of support given was trivial. Those *S. balanoides* occurring on *Littorina* had a regular shape, being small compared with the shell on which they sat.

Before the test was started, the length and breadth of the shell at the base were

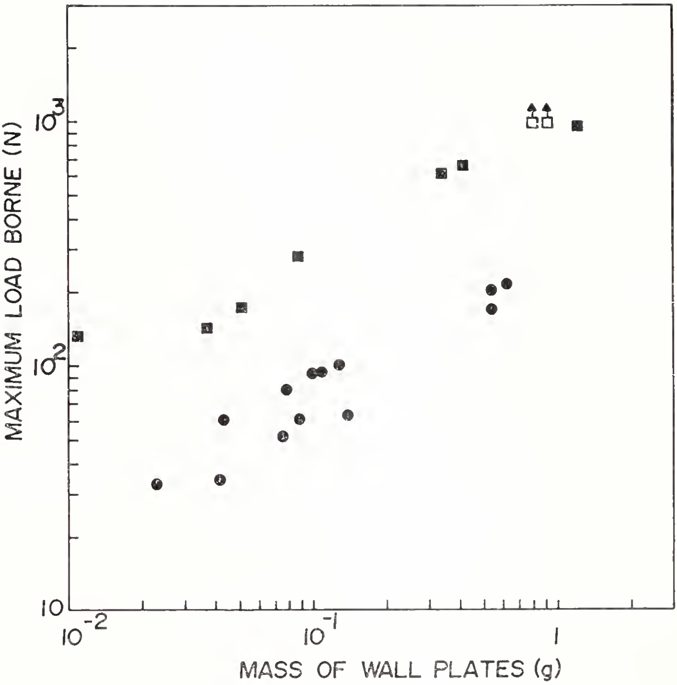


FIGURE 2. Shell strength expressed as the maximum load borne as a function of the mass of the shells for *B. balanus* (squares) and *S. balanoides* (circles). The two open squares indicate minimum values for two specimens of *B. balanus* which did not collapse under the maximum load which we were able to measure (1000 N).

measured. The height was taken from the base at a point mid-way along the rostro-carinal axis.

The head of the testing apparatus descended at 1 mm/minute, and the shells were loaded for a total of about a minute before they collapsed. The loading was usually not continuous; the head was moved up periodically to see what damage had been done.

The following measurements were made: maximum load borne; and the work done on the specimen up to the point of maximum load. The work was taken as the area under the load/deformation curve. This is the same as the energy absorbed by the specimen, the shaded area in Figure 1a.

After the test, the shells were cleaned for 24 hours in warm dilute KOH. The opercular plates were discarded, since they can have provided little strength in the mode of testing used. The shell plates were washed repeatedly and dried in an oven at 100° C before being weighed. The thin calcareous basis of *Balanus balanus* could not be weighed.

Figure 1a shows a typical load-deformation trace. This trace is idealized because usually the head applying the load was raised occasionally. When the specimen was reloaded, the load-deformation curve, instead of being jagged, usually rose smoothly up to the load that had been attained just before the head was raised.

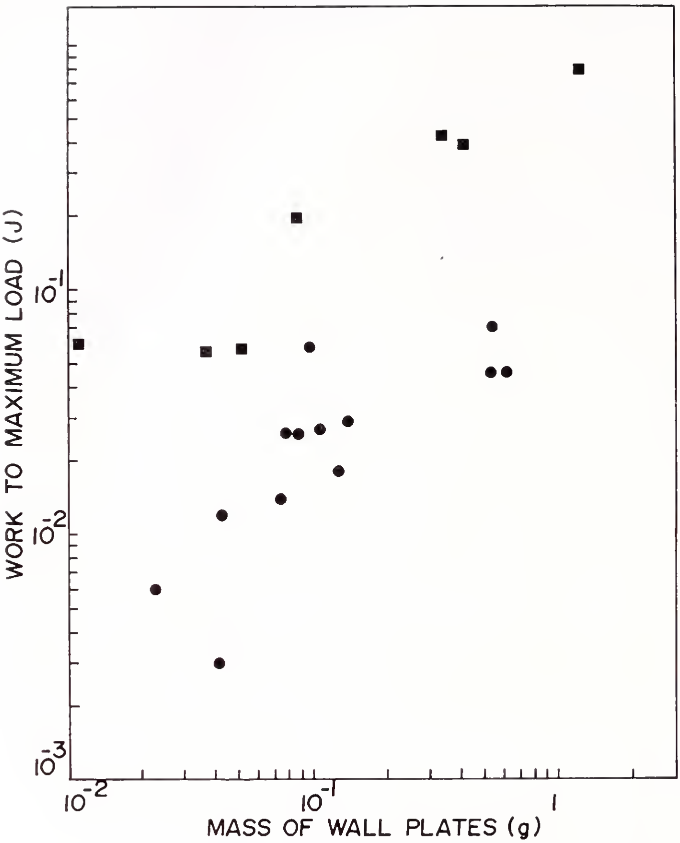


FIGURE 3. Shell strength presented as in Fig. 2 and expressed as the work done on the specimen up to the point of maximum loading (equal to the energy absorbed by the specimen). The two open squares indicate minimum values for two specimens of *B. balanus* which did not collapse under the maximum load which we were able to measure (1000 N).

This is reasonable evidence that the unloading-reloading cycle had not greatly altered the behavior of the shell. For purposes of calculating the energy absorbed, the area under the second curve up to the previously attained load was ignored (shaded in Fig. 1b).

Clearly, the larger the shell, the greater the load it should bear. Size was characterized in two ways: by the product of length and breadth and height of the shell, which we call pseudovolume; and by the mass of the dry, cleaned wall plates of the shell.

Scanning electron microscopy

Whole wall plates, fracture surfaces and etched polished surfaces were examined with the SEM. Polishing was accomplished with increasingly fine grades of carborundum paper and finished with alumina polishing paste on a felt pad. The polished

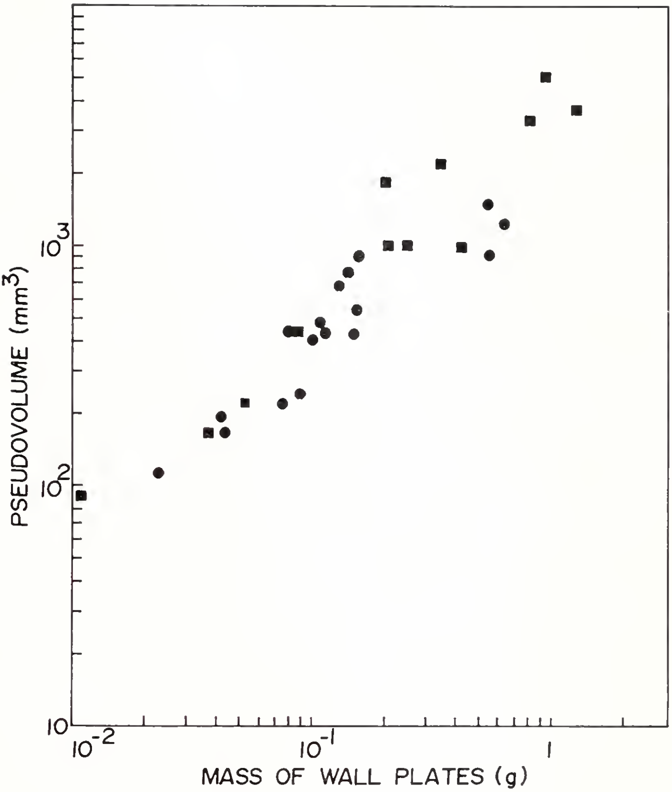


FIGURE 4. Shell pseudovolume (length × breadth × height) as a function of the mass of the wall plates for *B. balanus* (square) and *S. balanoides* (circle).

specimens were etched for a few seconds with very dilute hydrochloric acid. The specimens were then coated with gold.

Microhardness

Microhardness measurements were made with a Tukon microhardness tester which makes square-based pyramidal indentations with a diamond. The diagonals of the indentations were about 15 to 25 μm across, so differences between quite closely adjacent parts could be tested. The specimens were tested wet because dry mollusc shells, of similar composition, have been found to give higher values than wet ones (Currey, 1976).

RESULTS

Mechanical testing

The results of our mechanical tests show that for a given size, shells of *B. balanus* are consistently several times as strong as those of *S. balanoides*. This is

TABLE I

Results of compression tests on whole shells.

	Size (mm)			Shell Mass (g)	Max Load (N)	Work (J)
	Length	Breadth	Height			
Balanus balanus	19	17	7	0.341	618	0.433
	20	19	10	1.223	965	0.831
	18	17	17	0.916	>1000	—
	14	12	6	0.418	672	0.392
	18	21	9	0.796	>1000	—
	7	8	4	0.052	174	0.057
	7	6	4	0.037	144	0.056
	11	10	4	0.087	282	0.197
	5	6	3	0.011	132	0.060
Semibalanus balanoides	11	12	6	0.140	64	0.029
	11	12	5	0.129	103	0.018
	11	10	2	0.088	62	0.026
	10	11	4	0.099	93	0.058
	11	11	4	0.108	95	0.027
	10	11	4	0.078	80	0.026
	10	11	2	0.075	52	0.014
	17	16	10	—	390	0.136
	7	8	2	0.023	33	0.006
	7	8	3	0.043	61	0.012
	8	8	3	0.042	35	0.003
	16	13	6	0.626	219	0.046
	14	13	8	0.539	204	0.046
	13	12	6	0.540	180	0.071

true whether strength is expressed as the maximum load borne by each shell or as the work done on the shell by the applied load up to the point of maximum loading. Figure 2 shows the relationship between the maximum loads borne and the masses of the shells. Figure 3 shows the relationship between the work done by the applied

TABLE II

Relationship between dependent and independent variables. L is the maximum load in Newtons; V is the pseudovolume in mm^3; W is the work in Joules done up to the point of maximum loading; and M is the dry mass of the shell in grams.

Species	Relationship	F	d.f.	P	Coefficient of determination
B. balanus	$\log L = 0.95 + 0.571 \log V$	83.3	1, 6	$\ll 0.001$	0.94
S. balanoides	$\log L = 0.15 + 0.674 \log V$	48.2	1, 13	< 0.001	0.81
B. balanus	$\log L = 2.96 + 0.488 \log M$	91.2	1, 6	$\ll 0.001$	0.95
S. balanoides	$\log L = 2.44 + 0.561 \log M$	89.8	1, 12	$\ll 0.001$	0.89
B. balanus	$\log W = -2.89 + 0.781 \log V$	66.6	1, 6	< 0.001	0.93
S. balanoides	$\log W = -3.90 + 0.851 \log V$	25.3	1, 13	< 0.001	0.59
B. balanus	$\log W = -0.15 + 0.651 \log M$	38.9	1, 6	< 0.01	0.89
S. balanoides	$\log W = -1.01 + 0.697 \log M$	18.5	1, 12	< 0.001	0.63

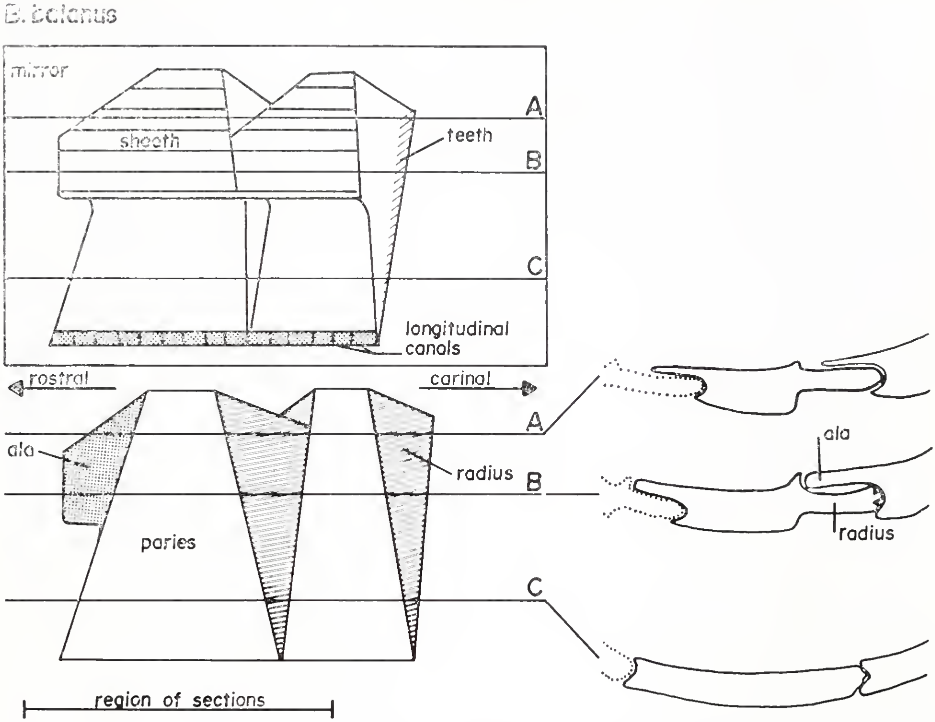


FIGURE 5. Stylized diagrams of two lateral wall plates from *B. balanus*. The outer aspect is shown below and the inner is shown above as reflected in a mirror. To the side are shown the outlines of frontal sections at various levels in the shell.

loads up to the points of maximum loading and the masses of the shells. Pseudovolume and mass are highly correlated (Fig. 4). Taking the values of *B. balanus* and *S. balanoides* together, the correlation coefficient is 0.96, and the shell mass is proportional to pseudovolume to the 1.13 power, showing that there is near isometry between shell mass and pseudovolume over the range examined. Because pseudovolume is highly correlated with shell mass, we have not provided figures on its relationship to shell strength. The dimensions of the shells, their masses, and the two measurements of strength for each are presented in Table I. Table II shows the linear regression relationships between the dependent and independent variables which appear in Table I together with the relevant statistics. The last column in Table II shows the coefficient of determination or goodness of fit for each regression. This statistic is never less than 59% for *S. balanoides* nor less than 89% for *B. balanus*. For both species, therefore, a large proportion of the variation in strength can be explained in terms of variation in shell mass or pseudovolume.

The forms of the load-deformation curves were generally similar between the species, resembling Figure 1. There was an initial steeply rising phase with a few small reductions in load caused by the breaking of the irregular peaks around the operculum. The crosshead of the Instron moves with a constant speed, so a

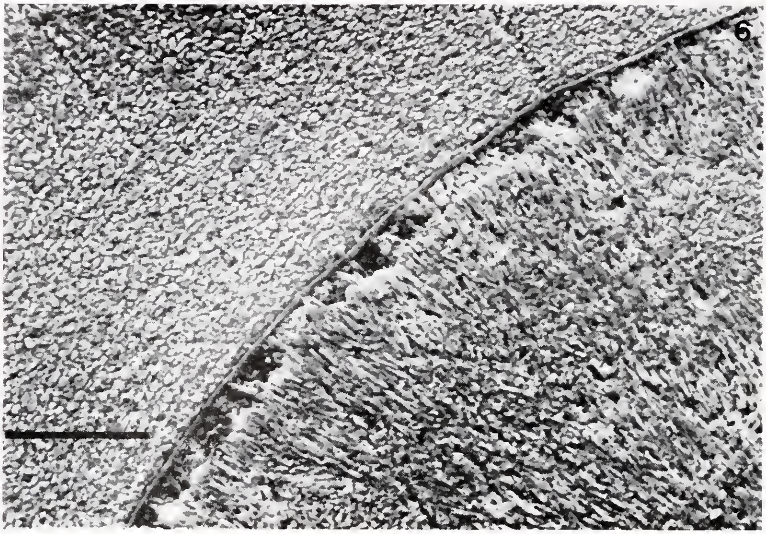
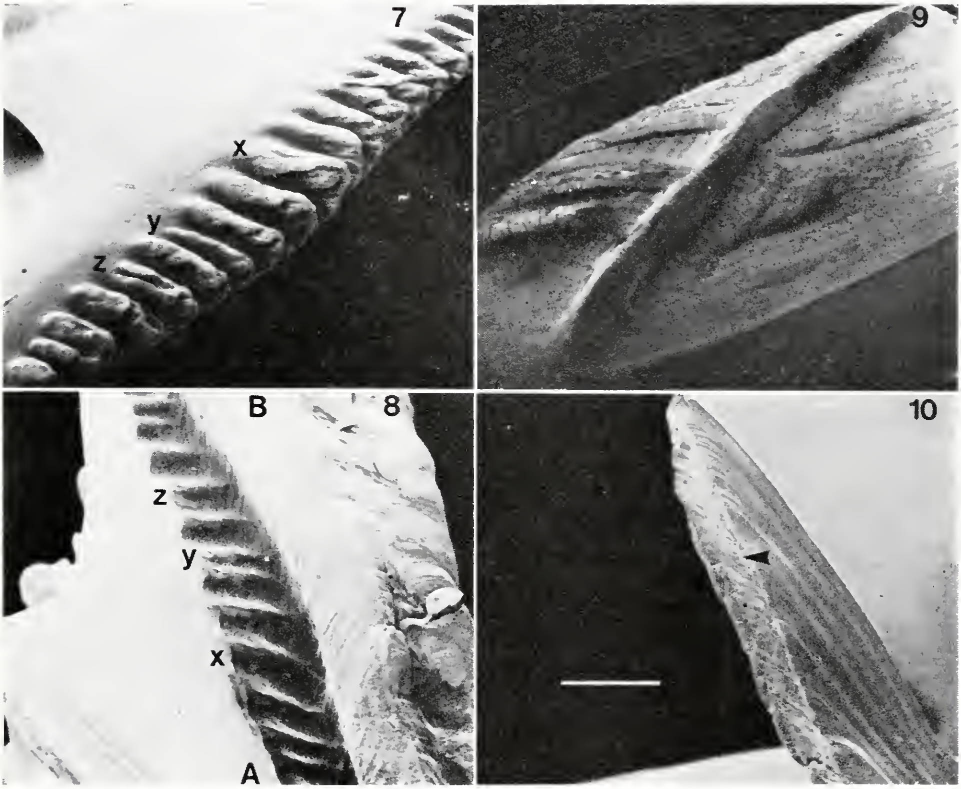


FIGURE 6. A scanning electron micrograph of the junction between the distal margin of a radius of *B. balanus* and the neighboring plate. A layer of cuticle can be seen between the two opposed surfaces. The section is parallel to the basis. The scale bar represents 20 μm.

crack in the specimen produces a sudden reduction in the load rather than a sudden increase in deformation. After the steep phase there was either a gently rising or a gently falling phase, again with frequent small reductions in load. The load sometimes declined slowly, nearly to zero, but at other times there occured a sudden large decrease in load; this was more often seen in *B. balanus* than in *S. balanoides.*

After the crushing down of the high points of the shell, one of the plates would eventually crack. Although the junctions evident between the plates would seem the natural place for the plates to fail, overt failure rarely began there; the first sign of failure was seldom the obvious moving apart of a pair of plates. Nevertheless, a slight movement of a junction, imperceptible to us, could throw an intolerable load on the neighboring plate by causing the load on the plate to be transmitted to the substrate at one place only. Usually failure of the shell would start with one of the plates cracking from top to bottom. It is not possible to tell whether the crack ran up or down. Once this happened the shell would start to disintegrate, with plates moving apart at the junctions and cracking, for the most part longitudinally. There was no really obvious difference in the mode of failure of the two species except that, once *S. balanoides* started to break, the plates seemed to move apart at the base more readily than did those of *B. balanus.*

Twice in testing large *B. balanus* (pseudovolumes of 3402 mm^3 and 5202 mm^3) the load exceeded the 1000 N limit of our instrument without destroying the shell being tested (Table I and Fig. 2). In each case, the top of the shell was greatly crushed, but damage did not extend down to the point where the hypodermis joins the shell wall. The crushed part of the shell had broken as a series of flakes approximately parallel to the inner surface of the sheath. The animals appeared unharmed, pumping and feeding normally when replaced in sea water.



FIGURES 7–14. Low power scanning electron micrographs of the wall plates of *B. balanus* and *S. balanoides* (at approximately the same magnification) showing the major features of the plates important in the formation of junctions with neighboring plates. Scale bars on Figures 10 and 14 represent 400 μm.

FIGURE 7. The ridged edge of the radius on a carinolateral plate of *B. balanus;* x, y, and z mark ridges which correspond to similarly marked pits on Figure 8.

FIGURE 8. Pitted groove along the edge of the paries of the carina of *B. balanus.* This receives the edge of the radius in Figure 7; x, y, and z indicate pits which correspond to similarly marked ridges in Figure 7. A and B mark the apical and basal ends, respectively.

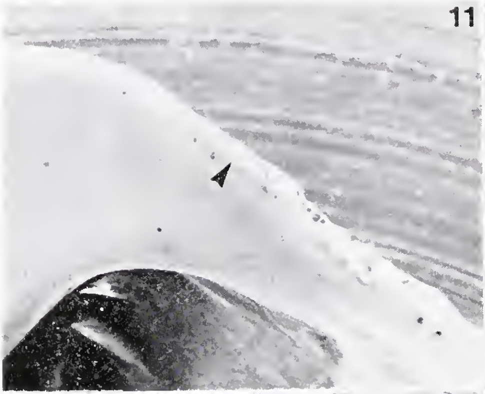
FIGURE 9. Groove in the sheath region of the carinolateral plate of *B. balanus* which receives the ala of the carina. Note that it is smooth.

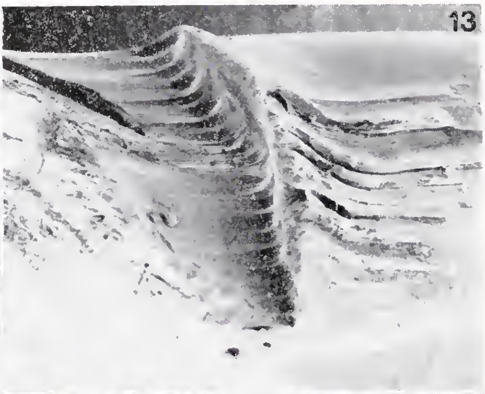
FIGURE 10. Rostral edge (indicated by arrow) of the ala on the carina of *B. balanus.* Note that it is fairly smooth.

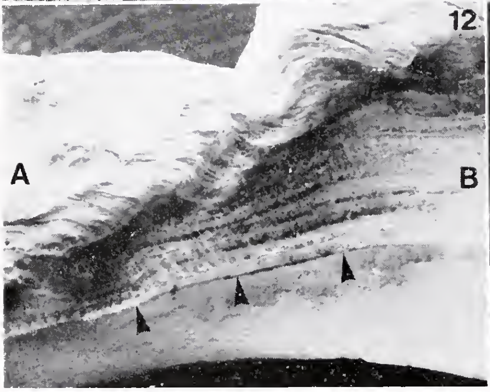
Morphology of B. balanus

Figure 5 presents a highly stylized representation of the inner and outer aspects of a pair of lateral wall plates of *B. balanus.* The perspective has been deliberately distorted to make the plates appear flat in order to emphasize certain morphological points which are not easily seen in drawings of more realistic perspective.

Each radius abuts the adjacent plate near its apex and is in contact with it for virtually the whole height of the shell (Fig. 5). The carinal margin of each radius is received snugly in a groove along the edge of the paries of the next plate, just







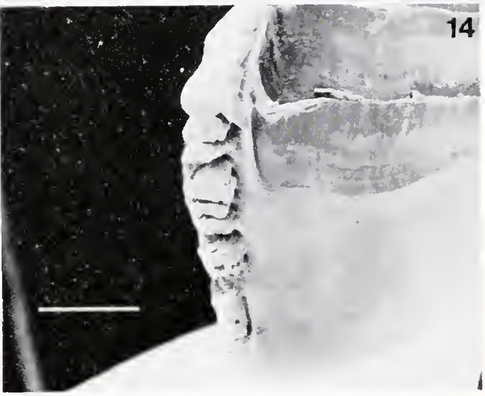


FIGURE 11. Inner surface of the radius on the carinolateral plate of *S. balanoides*. Compare its even edge (arrow) with the corresponding structure in *B. balanus* (Fig. 7).

FIGURE 12. Margin of the paries on the carinal plate of *S. balanoides* where the edge of the radius of the carinolateral butts against it. Contact begins along the line indicated by the arrows and extends as a snug abutment for a short distance only in the direction of the tails of the arrows. The region figured is a little below section A in Figure 23. A and B indicate the apical and basal ends of the structure, respectively.

FIGURE 13. Groove in the sheath region of the carinolateral plate of *S. balanoides*. The shallow pits receive the teeth on the rostral margin of the ala of the carina.

FIGURE 14. The roughly toothed rostral margin of the ala on the carina of *S. balanoides*.

outside the base of the ala. (Snug is defined here as the condition in which the two plates fit tightly up against each other, with the interposition of only a very thin layer of organic material, a few microns thick; Fig. 6.) The inner surface along the radius is ridged (Figs. 7, 15), the ridges projecting somewhat beyond the end as teeth. These ridges are probably basically the same as those figured by Darwin (1854) for *B. tintinnabulum* [*Megabalanus tintinnabulum* (Newman and Ross, 1976)] and strikingly similar (especially in Darwin's figures) to the teeth at the base of the paries which give rise to the so-called interlaminate figures (Newman, Zullo and Wainright, 1967). These ridges fit into shallow pits in the bottom of the groove on the paries, which groove receives the edge of the radius (Figs. 8, 16). The tips of the ridges often have a chalky white appearance. The radius doubtless continues to grow towards the carina along this margin, this growth having the effect of pressing the ridges into their pits and widening the opercular opening of the shell.

The alar margins slope from the apices of their plates rostrally and basally until they abut a ridge running perpendicular to the basal margin of the sheath on the next more rostral plate. This ridge often forms a shallow groove (Figs. 9, 17) which receives snugly the rostral margin of the ala much as the carinal margin of the radius is received. The ala, unlike the radius, is not strongly ridged though the rostral edge may be slightly knobbly or lumpy (Figs. 10, 18). The material forming the rostral edge of the ala is chalky white, very reflective and opaque both when seen as a complete plate, and in polished sections. The rest of the plate material is substantially more translucent.

Between the overlapping radius and ala, in the mid-region of the overlap when viewed in section (as in Fig. 5), is a narrow space. Smaller spaces occur near the ends of these projections where they form snug abutments with neighboring plates. Except in their upper regions these spaces contain tissue in life (see Costlow, 1956).

Below the level of the sheath, the radius still forms a ridged projection on the carinal margin of each wall plate. This is received in a matching shallow pitted groove on the abutting plate. Along the base of the plate, the bases of the longitudinal septa form teeth which interlock with holes on the calcareous basis, formed at the ends of radiating canals, as described by Darwin (1854), and Newman *et al.* (1967). There is a thin layer of tissue between the plates and the basis.

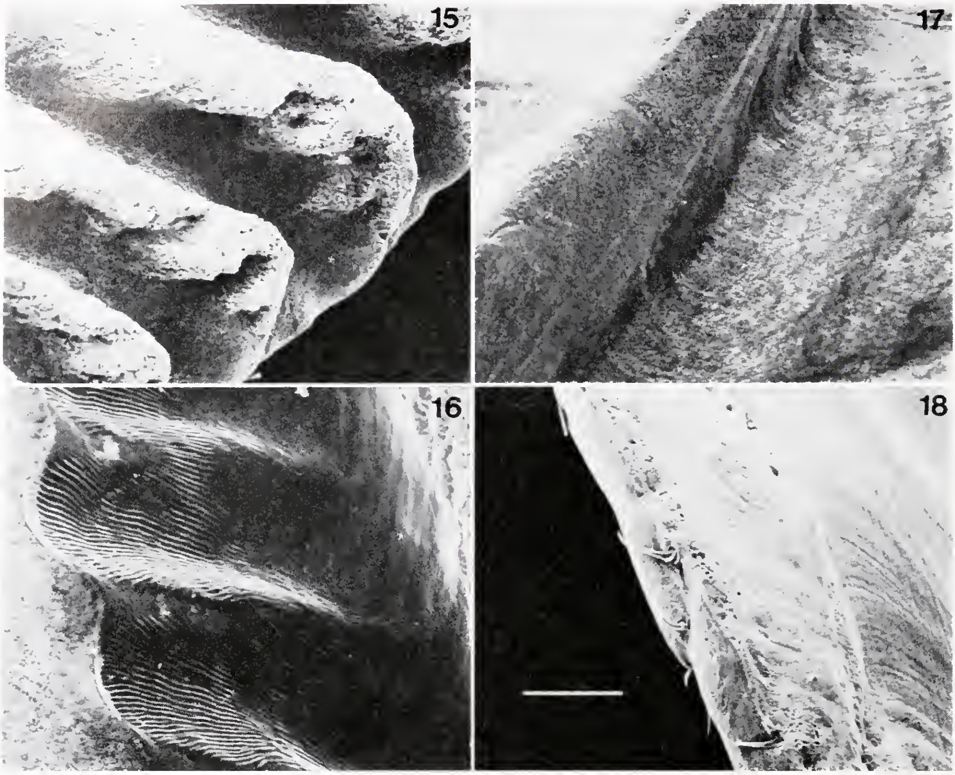
Morphology of S. balanoides

Figure 23 presents a stylized view of two plates from the shell of *S. balanoides,* drawn in the same manner as Figure 5. More realistic drawings of the wall plates of *S. balanoides* are to be found in Stubbings (1975). Other diagrams are available in Gutmann (1960).

Compared with *B. balanus,* the radius is much less prominent, forming little more than a narrow strip along the carinal edge of the paries (Figs. 11, 19). The inner surface, near the edge of the radius, may meet the outer surface of the underlying ala in a flat junction, but the margin of the radius is typically not received in a groove except perhaps very near the base of the shell (Figs. 12, 20). This is in marked contrast to *B. balanus.*

It appears from our sections that the carinal margin of the radius is often not in close contact with the ala though this may possibly be an artifact. The inner surface of the radius consists of chalky white material similar in appearance to that described above on the edge of the ala in *B. balanus.*

The ala is very much stouter than the radius. It is roughly square-ended both in profile (inner or outer surface) and in section. The rostral margin abuts the next shell snugly over much of the height of the sheath region. This is in contrast to *B. balanus* where only the lower part of the alar margins abuts the neighboring plate. The portion of the rostral margin nearest the overlying plate is again chalky white and lumpy, and may have some teeth (Figs. 13, 14, 21, and 22). In the central part of the overlap between the ala and the overlying portion of the neighbor-



FIGURES 15–22. Higher magnifications of the areas shown in Figures 7–14. The scale bars on Figures 18 and 22 represent 100 μm.

FIGURE 15. Edge of the carinolateral radius of *B. balanus*.

FIGURE 16. The pits in the groove on the carina of *B. balanus* which receive the radius of the carinolateral. Note that the pits themselves have ridges.

FIGURE 17. Groove in the sheath of the carinolateral plate of *B. balanus* which receives the ala of the carina.

FIGURE 18. Rostral edge of the ala on the carina of *B. balanus*.

ing plate is a substantial gap except in the areas of snug fit mentioned above. Again, as in *B. balanus*, except in its upper part, this gap is filled with tissue in life.

Below the sheath, the wall plates butt up tightly against each other, usually showing little sign of geometric locking devices. There is no calcareous basis, and the plates fit down against the substratum with only the thin membranous basis intervening.

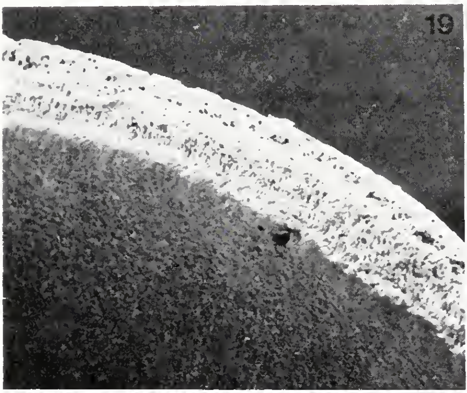
Shell microstructure

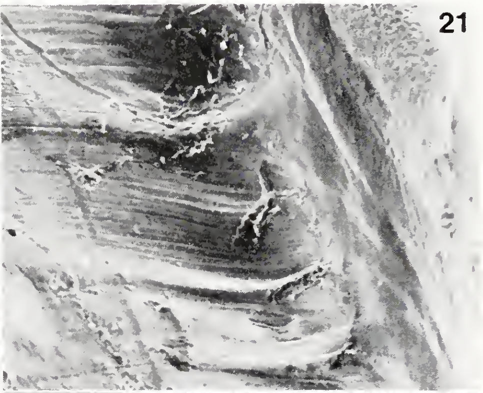
Most of the material of the etched specimens of both species appeared very similar to that which, in molluscs, is termed 'homogeneous' (Taylor, Kennedy, and Hall, 1969). This is characterised by roughly isodiametric grains which, in these barnacles, are of the order of 1 μm in diameter. It is quite possible, of course, that these "grains" may be polycrystalline, but our scanning electron microscope could not resolve better than about 0.1 μm. In places, in the radius and ala of *B. balanus* and in the ala of *S. balanoides*, there are elongated crystals of the order of 1 μm in diameter and more than 10 μm long. These form fan-like arrays reminiscent of spheritic growth forms in scleractinian corals (Bryan and Hill, 1941; Jell, 1974). Where they occurred near the surface of the plate they were approximately perpendicular to it (Fig. 6). These arrays of long crystals frequently show transverse banding, probably indicative of variations in growth rate.

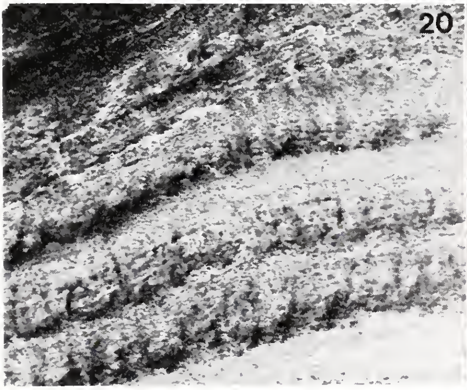
The chalky marginal areas of the wall plates, which are shown below to be soft, are very fine-grained (grain size approximately 0.5 μm) while the harder translucent areas show the normal larger grain size characteristic of the rest of the plate.

Microhardness

Tests were carried out both in the vicinity of junctions in the sheath region and on the parietes. Readings taken *very* close to a junction or an edge tended to







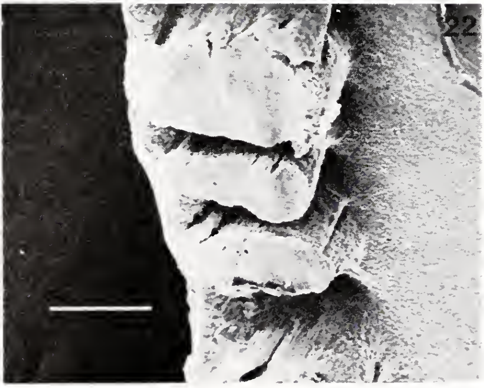


FIGURE 19. Edge of the radius on the carinolateral plate of *S. balanoides*.

FIGURE 20. Region on the carina of *S. balanoides* where the radius of the carinolateral plate butts against it.

FIGURE 21. Shallow pits in the groove in the sheath region of the carinolateral plate of *S. balanoides* which receive the teeth on the rostral edge of the ala of the carina.

FIGURE 22. Teeth on the rostral edge of the ala on the carina of *S. balanoides*.

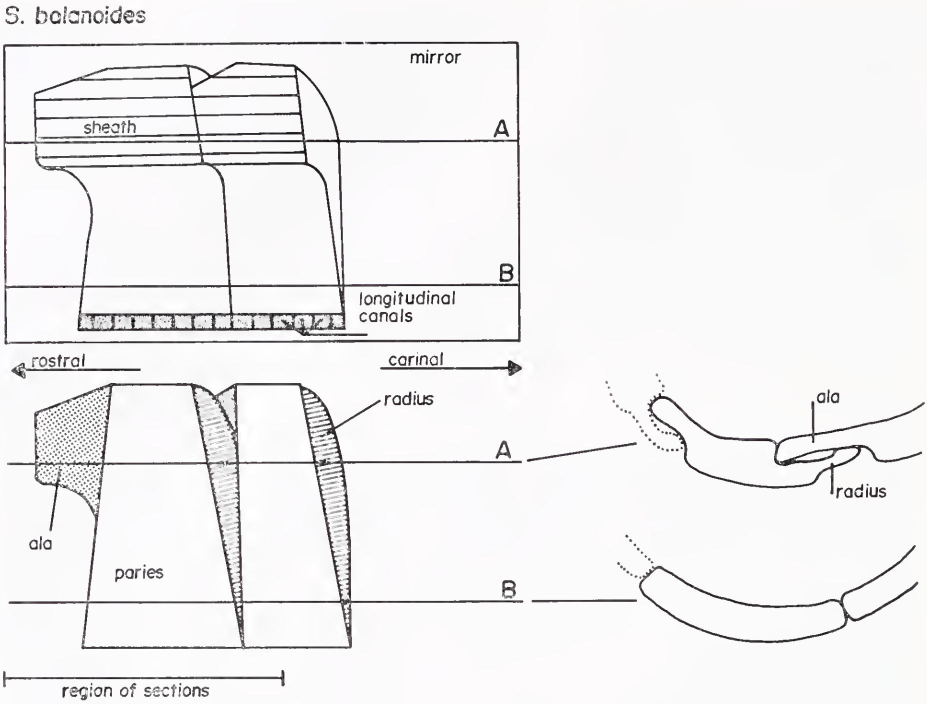


Figure 23. Stylized diagram of two lateral wall plates from *S. balanoides,* presented as in Figure 5.

be lower than elsewhere, probably because the proximity of a free surface to one side of the indentation allows the material to deform more easily in that direction. Therefore, readings taken within about 50 μm of a junction are excluded from these results.

Tests were made on surfaces in three different orientations: parallel to the plane of the basis (frontal); tangential to the shell as a whole (tangential); and radial to the shell as a whole (radial). These last tests were an attempt to measure the hardness of the junctions in the direction in which they would be loaded by the adjoining plate.

Microhardness tests were done on ground and polished frontal surfaces of the parietes in the sheath region for all six plates from one specimen of each species. The results are given in Table III, denoted by "paries far from junction". The means are not significantly different. Even if the difference between the means were real, it would suggest quite trivial differences in the mechanical properties of the shell materials.

In some places, usually close to junctions, the shell was chalky white instead of translucent or grey, as it was in other places. We expected that there might be differences in michrohardness accompanying these optical differences. In tangential and frontal surfaces it was difficult to ensure, first, that one was not loading a

TABLE III

The hardness of shell material in various regions and various orientations.

Species	Part	Orientation	Mean hardness	n	s.e.
B. balanus	Paries, far from junction	Frontal	245.9	32	5.03
	Paries, near junction	Frontal	276.6	11	6.25
	Radius, near junction	Frontal	253.6	16	11.00
	Radius, toothed edge	Radial	223.5	20	6.6
	Groove on paries receiving radius	Radial	217.3	16	6.5
	Anterior edge, ala (chalky)	Radial	113.2	9	11.3
	Anterior edge, ala (translucent)	Radial	215.3	7	8.6
	Posterior edge, radius	Tangential	231.7	10	10.0
	Paries (where radius butts against it)	Tangential	242.0	7	14.3
S. balanoides	Paries, far from junction	Frontal	235.5	30	3.0
	Paries, near junction with ala	Frontal	235.1	7	11.5
	Ala, near junction with paries	Frontal	242.3	6	13.5
	Distal edge of radius	Radial	173.8	8	10.5
	Anterior edge, ala (chalky)	Radial	199.2	10	6.1
	Anterior edge, ala (translucent)	Radial	323.5	6	6.7
	Anterior edge, ala	Tangential	202.6	10	9.8
	Paries (where ala abuts)	Tangential	245.7	10	14.6

chalky region that was in fact overlain by, or overlying, a thin layer of translucent material and secondly, that one was not loading very close to an edge of the chalky material.

Therefore, we have not distinguished between chalky and translucent regions in tests on frontal and tangential surfaces. On radial surfaces, however, the differences in microstructure were clear, and the two are distinguished in the results presented in Table III.

In general, the microhardness values varied from region to region in a not very predictable way (Table III). The one consistent difference was that on radial surfaces the chalky region was softer than the translucent region next to it. An example from each species is shown in Figure 24, with the locations and values of the hardness tests indicated. The rather high values for *S. balanoides* are unusual, not being seen elsewhere in either species.

DISCUSSION

The shells of *B. balanus* are about three to thirteen times as strong as those of *S. balanoides,* depending on the variables considered. This difference in strength is very unlikely to be due to the mechanical properties of the materials in the shells of the two species and is probably due to differences in the large-scale architecture of the wall plates and bases of the shells.

The differences in the bases provide the most obvious, and possibly the most significant mechanical difference between the shells of the two species. *S. balanoides* shells are kept on the substratum merely by the strength with which they are attached to the membranous basis, which is in turn firmly glued to the substratum (Stubbings, 1975). In *B. balanus,* although there is tissue between the wall plates

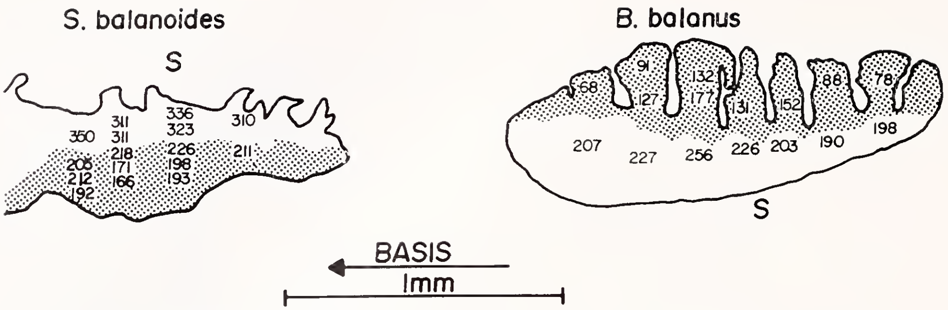


FIGURE 24. Radial sections (perpendicular both to the basis and to the shell wall) at the distal margin of an ala from each of *B. balanus* and *S. balanoides*. The stippling indicates the opaque white areas in the specimens, unstippled areas were translucent. The numbers are microhardness values and each is centered on the location of the test it represents. The letter S indicates the sheath side of the section. In each case, the basis of the shell is to the left, and the apex to the right.

and the calcified basis, there is a fairly complex system of interlocking buttresses (Newman *et al.,* 1967) that anchor the shell to the basis which is firmly glued to the substrate.

There is a well-known tendency of *S. balanoides* to grow in serried masses, with a resulting likelihood of peeling off the substrate in great sheets (Connell, 1961; Barnes and Powell, 1950). It is possible that the mechanical importance of the basis of *S. balanoides* should be considered in this context. The lack of a calcified basis will be less important, from the point of view of resisting direct loading, in an animal which is supported on all sides. Calcified bases will help stop the feet of the plates from slipping outward as the result of loads from on top. In *S. balanoides* (or in any other species) if the feet of the plates are jammed up against adjacent shells, the lateral support function may be taken over by those shells. This does not mean, of course, that a calcareous basis would not be useful. It could be that, for isolated individuals, and even in massed individuals, a more-or-less common calcified basis would help prevent the whole mass from being dislodged.

The wall plates differ between the two species in a characteristic which seems best described as workmanship. Workmanship is defined here as regularity and precision in the details of construction, particularly in the design of the joints between plates.

There is a difference in workmanship between the two species which is particularly clearly visible in uncrowded specimens. This is that the external form of *B. balanus* is much more regular than that of *S. balanoides*. *B. balanus* has a neat volcanic shape with sharp radiating ridges. Growth arrest lines are not readily visible. *S. balanoides* is, in general, lumpy, with clear growth arrest lines and overhangs. Whether these differences are mechanically important cannot be proven, but irregularities such as overhangs and bumps contribute little to the strength of a structure, and could even create local stress concentrations, weakening it. Although, when loaded slowly, the shells of these barnacles seem not to come apart first at the junctions between the plates, it is likely that the integrity of the junctions is of paramount importance to the animals. Barnes *et al.* (1970) claim that they do come apart first at the junctions when loaded laterally. Effectively any kind of loading will, because of the irregularities in the shell, tend to make some of the wall plates shear past each other at their junctions. If the junctions are rigid, the shell will continue to bear the load as a whole. If, however, the junctions have some play, they will allow the plates to rock slightly relative to each other and relative to the substrate. This rocking will produce very high local stresses, often at points well removed from the junctions.

The difference between the two species in the strengths of the junctions between wall plates was qualitatively obvious. During the preparation of specimens of the two species for sectioning and microscopical examination, there was a much greater tendency for *S. balanoides* simply to fall apart at the junctions than for *B. balanus* to do so, even when the calcareous basis of *B. balanus,* which gave considerable coherence to the shell, had been removed.

The distal margin of the radius of *B. balanus,* where it abuts on the neighboring plate, is received in a groove (Fig. 5). Such a junction (which we shall call a *dado,* as do Barnes *et al.,* 1970), restricts possible disarticulating movements of the two opposed surfaces in the plane of a frontal section to one direction only, roughly parallel to the ala itself. Disarticulating motion in any other direction must break one of the two plates. So long as the opposing surfaces of the junction are held together, this will be a very strong type of junction.

A dado joint at the carinal margin of the radius of a plate of *B. balanus,* and the strong teeth on ridges along that margin, make the junction between that and the neighboring plate a strong one. The teeth increase the surface area of contact for adhesion and also prevent slippage parallel to the junction. Our scanning electron microscope results show a cuticular layer between the surfaces at this junction which may act as an adhesive, and probably seals the junction against invading organisms (Fig. 6).

The carinal margin of the radius in *S. balanoides,* on the contrary, contacts the paries of the neighboring plate along its basal portion only, where it makes a flat or very shallowly-grooved junction. Therefore, the junction must be much weaker than the equivalent one in *B. balanus.* The upper margin may in places be closely opposed to the outer surface of the underlying ala and this region need not be thought completely devoid of strength. There is, however, no mechanical interlocking of the radius and ala. Therefore, the construction of the radial-parietal junction is much more precise in *B. balanus* and resists relative movement of neighboring plates by its geometrical arrangement. This is in strong contrast to *S. balanoides.*

The general form of the junction between the distal edge of the ala and the neighboring plate is similar in the two species. The length over which the junction is snug when compared with the height of the sheath region is somewhat greater in *S. balanoides* than in *B. balanus.* However, when the ala of *B. balanus* forms a snug junction it is often a snug dado, whereas the homologous junction in *S. balanoides* is never of this geometrically locking type.

In a sense the morphological pattern in the sheath region of the junction between the plates of *B. balanus* is the reverse of that in *S. balanoides* [compare frontal (horizontal) sections in Fig. 5 level B and Fig. 23 level A]. In *B. balanus* the strong, interlocking part of the junction is formed by the radius. In *S. balanoides* it is formed by the ala and, hence, cannot be longer than the sheath and is usually rather less. In comparing the strong parts of these two junctions, it is apparent that the margin of the radius of *B. balanus* always forms a good dado, geometrically interlocking joint, while the square-ended margin of the ala of *S. balanoides* usually does not. In *S. balanoides* the radius, and in *B. balanus* the ala, may form snug junctions along their distal margins but not such strong ones.

We can only speculate why the radius or the ala should be preferred for the strong part of the junction. There is no obvious important mechanical reason to prefer one design over the other in the sheath region, although, of course, the strong toothed and ridged radial joint of *B. balanus* extends below the sheath region. All else being equal, the more exposed surface of the junction seems more likely to be attacked by organisms. Thus a snug junction at the margin of the radius may be very important, not only for mechanical reasons, but also in preventing the ingress of potentially destructive organisms.

The outer surface of *S. balanoides* is often badly corroded by weather and abrasion through wave action, common in the high energy intertidal environment. To put a strong junction on the outward facing surface (the sheath, too, is actually external to the mantle cavity and exposed to the environment) may be to put it where it is likely to be destroyed no matter how well designed and constructed. Natural selection may have favored placing the stronger junction at the inner surface of the sheath region in *S. balanoides* to shield it from damage.

Junctions may be held together by adhesives as well as by their geometry. There is evidence of a very thin layer of organic material, presumably adhesive, in some junctions (see Fig. 6). In general, junctions held together by adhesives are strong only if the two glued surfaces are closely apposed (Gordon, 1976). This may be an important aspect of the mechanical design of these junctions which we refer to as "snug." Bocquet-Védrine (1965) claims that extensions of the cuticle of the animal may serve as an adhesive in some junctions.

B. balanus, therefore, is consistently more regular and precise of construction than is *S. balanoides; B. balanus* has superior workmanship. This greater regularity and precision must contribute to the greater strength seen in the shells of *B. balanus.*

The resistance of *B. balanus* shells to crushing was strikingly shown in the two specimens which resisted 1000 N by the flaking of the top of the sheath rather than cracking. This may represent a mode of nondisastrous, controlled collapse which allows large animals to absorb the energy of impact without failure. Additionally, such flaking may increase the surface area bearing the load, thereby decreasing *local* stresses and the likelihood that a disastrous crack will start.

Although the shells of *B. balanus* and *S. balanoides* differ in their construction and workmanship in a way that might account for the observed differences in strength, it is also possible that differences in the mechanical properties of the shell material itself might make a substantial contribution to the differences in the properties of the whole shells. Differences in the mechanical properties of molluscan shell materials are due to differences in the microstructure of those materials (Currey, 1976, 1977; Currey and Kohn, 1976). The constitution of barnacle shells is similar to that of molluscs (Bourget, 1977; Barnes, Klepal and Mitchell, 1976; Wilbur and Simkiss, 1968). Thus, if the observed large difference in strength between the two species is due to differences in material properties, a significant difference in microstructure should be seen between the two.

It is remarkable, therefore, that the macroscopically and mechanically dissimilar shells of these two species are so very similar at the microstructural level. It is very unlikely that two materials so similar in microstructure should differ sufficiently in mechanical properties to explain the overall differences in strength which were measured.

Despite the ultrastructural similarity of the materials in the plates in the two species, we wanted to measure and compare the mechanical properties of the plate materials. Because the plates themselves are of such irregular shape, we judged conventional compression, tension or bending tests unlikely to yield useful results (see Wainwright, Biggs, Currey and Gosline, 1976). Hardness tests, however, were possible on small, polished specimens. Hardness is not a well understood feature of materials. It is not easily related to other mechanical properties, but it seems to depend on the modulus of elasticity and the yield strength in compression (Mott, 1956). Thus, if the observed difference in overall strength between species was largely attributable to differences in mechanical properties of the materials of the shell of the two species, this difference should be reflected in microhardness tests. The values given by the tests can be considered as arbitrary, as we are interested only in comparisons between the two species.

Although mechanically important variations in hardness within the shells may be demonstrable, there is no general difference between the species which could explain the observed differences in overall strength. This is again remarkable in light of the difference in overall properties of the two shells, but, like the ultrastructural results, argues strongly that the observed differences in shell strength are not due to differences in the properties of the shell material.

In lateral impact loading Barnes *et al.* (1970) found that *B. balanus* was stronger that *S. balanoides*, though only by about 50% or so at the greatest, and they indicate (their Fig. 2) that at small sizes *S. balanoides* is slightly stronger than *B. balanus*. They performed some impact tests on cantilever test pieces cut from shell plates, and could find no large difference in "momentum per unit area" resulting in fracture. They conclude (p. 82) that "since the strength of the wall plates *per se* for animals of the same size has been shown to be similar any distinction between the species must arise as a result of differences in strength at the junctions." This seems a rather sweeping conclusion, since a number of other things could affect the strength of the whole shell: the shape of the plates, the shape and construction of the whole shell, and the mode of attachment of the shell to the substrate. We agree with Barnes *et al.* (1970) that junction morphology is an important factor in determining junction strength. Their means of characterizing the morphologies of the junctions by describing sections of the shells taken at "mid height" is likely to lead to erroneous conclusions, however. It is clear from their published pictures that they are comparing junctions from the sheath region of *S. balanoides* with those from below the sheath in *B. balanus*. Inspection of the disarticulated wall plates will show that an understanding of the shape of the junction between two plates is possible only with sections from at least two levels (Figs. 5 and 23): in the sheath region and below it. Also, uneveness of the substratum often results in asymmetry in the growth of a barnacle. Unless care is taken under such conditions, a section taken through the shell near mid height is likely to pass through two distinct regions of the shell which differ markedly in their morphology. Several such sections are presented by those authors; in particular, the sections of *Chthamalus stellatus*, *Semibalanus balanoides* and *Balanus crenatus* pass partially through the sheath and partially below.

Our results cannot be compared directly with those of Barnes *et al.* (1970). They determined the height from which a metal load of known mass must be dropped just to break the shells and calculated the approximate momentum of the bar at the moment of impact, whereas we loaded slowly. Furthermore, we loaded the specimens on top, whereas they loaded them from the side. While they indicate the range of sizes available for testing for each species in their Table 1, several of the regression lines in their Figure 2 extend beyond the ranges given. Thus, it seems clear that they did not determine that small (*ca.* 2.5 mm diameter) *B. balanus* and *S. balanoides* have the same strength as their Figure 2 indicates, since the smallest *B. balanus* available was 3.5 mm in diameter. The largest whole shell tested for each of these two species was 12 mm in diameter. As *B. balanus* commonly gets substantially larger than this, their sample does not appear to be representative of the species. They tested individual wall plates from *S. balanoides* 16–23 mm in diameter and from *B. balanus* 15–26 mm in diameter. It is unfortunate that some of these large specimens were not used to test the strength of whole shells. The number of specimens tested is not given, and so we cannot calculate the probabilities associated with the correlation coefficients they give, which are all very high. Barnes *et al.*, used high-speed ciné photography on a specimen of *S. balanoides*, which it is claimed shows that the junctions open before the plates crack. This is possibly the case, although the situation is by no means clear.

Given these various facts, it is not certain whether, for instance, *B. balanus* was determined by Barnes *et al.* (1970) to be weaker than *S. balanoides* at lower sizes as the crossing over of the regression lines would indicate, and whether the differences we observed in the higher size ranges were seen by them.

Since, when tested by our method of loading, *B. balanus* is so much stronger than *S. balanoides*, it is perhaps appropriate to speculate what selective forces have led to the differences between the species. It should be emphasized that we know the strength of the two species from our locality and from particular tidal levels, and although much of the information we have obtained from the literature refers to similar locations, it is possible that the strength of the animals, and their condition of life, may be different elsewhere.

Our hypothesis is that once settled, *S. balanoides* is likely to be killed in the first or second season by an event unrelated to shell strength. *B. balanus*, on the other hand, appears to live, once established, for several seasons and hence is more likely to encounter situations in which shell strength is critical to survival. *S. balanoides*, therefore, channels its energies early, as early as the middle of the first year, to reproduction at the expense of other factors such as the workmanship of the shell. *B. balanus*, on the other hand, begins to reproduce later, typically in the second year, and so invests more heavily in a secure shell to help ensure a prolonged survival.

The energentic cost of building a calcified skeleton is, unfortunately, unknown. If a high degree of order is needed in the deposited calcite, precise control processes will be needed which are likely to be energetically costly. For this reason, precise architectural construction may be costly for barnacles, and may be a significant fraction of the whole cost of building the shell.

In Britain *S. balanoides* is almost entirely intertidal, and *B. balanus* is sublittoral. The work of Grant (1977), Connell (1961), and Barnes and Powell (1950) shows that *S. balanoides* undergoes great fluctuations in population density. These fluctuations are produced by, among other things, overcrowding, which causes great sheets of consolidated shells to break off the substrate during storms (Barnes and Powell, 1950), depredation by whelks (Connell, 1961), sea ice, though rarely in Britain (Barnes, 1957), and even, for high littoral forms, calm hot days during neap tides (Connell, 1961). The result of these various sources of catastrophic death is that, from autumn to autumn, one may find patches of rock sometimes covered by *S. balanoides* approaching sexual maturity, and sometimes bare of barnacles. *B. balanus* rarely shows the gross overcrowding shown by *S. balanoides,* and it is almost certain that its environment, in British waters, is more stable from year to year.

In Britain *S. balanoides* shows little growth after one or two years (Barnes and Powell, 1953). Furthermore, the population of large *S. balanoides* is limited by predation by the whelk *Nucella lapillus,* which preys heavily on them in preference to smaller individuals. *B. balanus*, on the other hand, grows considerably after the first two years (Crisp, 1954). Data presented by Crisp indicate there is no sharp rise in mortality at any time during the first four years of life. Moreover, *B. balanus* does have a behavior pattern (Darwin, 1854; Crisp and Southward, 1961) which probably serves as an anti-predator device. The operculum can be tipped up and pressed against the sheath of the rostrum and lateral plates and rubbed from side to side. Any intruder who pokes at the opercular membrane is likely to be pinched between the opercular plates and the inner surface of the sheath. If *S. balanoides* is attacked, its only recourse is to close its opercular plates tightly. Being subtidal, *B. balanus* must have a shell capable of resisting attack from predators, such as crabs, which are less prone to venture into the high energy intertidal region (Kitching, Muntz, and Ebling, 1966).

Data derived from Barnes, Barnes, and Finlayson (1963) seem to indicate that *B. balanus* and *S. balanoides* produce a similar relative mass of sperm per season; the drop in body mass in the two species caused by the shedding of sperm (*ca.* 50%) is about the same (the "body mass" does not include the mass of ovarian tissue). However, Barnes and Barnes (1968) have calculated that the mass of eggs per unit body mass is about twice as great in *S. balanoides* as in *B. balanus.* This difference, though large, is subject to considerable experimental error, so should not be taken as very secure.

Calculations from data given in Figure 5 of Barnes *et al.* (1963) indicate that *B. balanus* grows a shell about two and a half times more massive, per unit dry body mass, than that of *S. balanoides.* This fits very well with our general view regarding the strength and general strategy of the two species. However, as Barnes *et al.* (1963) state, and Dr. Barnes (personal communication) has emphasized, these animals were grown under optimal conditions on rafts, and may not be very good indicators of the natural state of affairs. Furthermore, we performed some preliminary experiments in which the dry mass of the shell (excluding the calcareous basis of *B. balanus*) was compared with the dry body mass (excluding the ovaries). These measurements were taken in early July, 1977; the animals came from situations in the Clyde Estuary similar to those of animals tested mechanically. There was an indication that in the smaller animals, with dry body mass of less than 5 mg, *B. balanus* had shells slightly more massive than those of *S. balanoides,* but that this difference disappeared when body weight reached about 10 mg.

If the actual masses of the skeleton per unit body mass are approximately the same, would it not be possible for *S. balanoides* to build as strong a shell as *B. balanus* and yet incur no penalty? Although there are good reasons for thinking that a strong shell might often be of little use to *S. balanoides,* it would surely be useful on occasion, and if no increase in cost were associated with a stronger skeleton, it would be selected for. Even if the weight of the basis were included in the calculations of shell weight, the skeleton of *B. balanus* would be so much stronger that selection in the direction of a *B. balanus* type of skeleton on the part of *S. balanoides* would seem to be of advantage.

Two possible explanations can be proposed as to why no such selection has occured. First, the shell of *S. balanoides* may be designed to meet criteria other than strength in resisting the sort of load we applied, perhaps having to do with growth under extreme crowding. The lack of a calcareous basis in *S. balanoides* may be related to its habitually crowded living conditions. Secondly, the cost of precise, strong shell construction may simply be greater than is warranted given the ecology and life history of these animals. If precise construction is expensive, and if early reproduction in *S. balanoides* is essential, the observed shell design would make sense.

The superior mechanical performance of *B. balanus* shell is attributable to the design and precision of construction of the skeleton, and to the calcareous basis but not, insofar as these microstructural and microhardness studies give any indication, to differences in the shell material itself. Unfortunately, there is no quantitative information on the biological cost of building something precisely. Sometimes time is a cost. In many molluscs relatively weak, prismatic material is laid down first at the rapidly growing margin of the shell and is then slowly reinforced with the stronger nacre (Taylor *et al.,* 1969; Yonge, 1953). In vertebrates, rather disorganized woven bone is produced quickly in areas of rapid bone growth, to be replaced more slowly later by more highly organized lamellar bone (Wainwright *et al.,* 1976). In these examples rate of growth is apparently related to skeletal organization, but the relationship remains, of course, to be demonstrated in barnacles. In the natural habitat, the shells of the two species grow at similar rates, but *B. balanus* is slower than *S. balanoides* in the early summer of the first year (Barnes *et al.,* 1963). Because of the microstructural similarities of the materials in the shells of the two species, this difference in growth rate is probably not due to a difference in the cost of shell material. The early rapid growth by *S. balanoides* may, however, be necessary to obtain the minimum size for reproduction during the first year, and it may take place at the expense of the workmanship and strength of the shell. In the first autumn *S. balanoides* is engaged in reproduction and *B. balanus* catches it up in size, presumably because it can devote relatively more effort to growth.

GRM was supported by the Cocos Foundation. JDC was supported by the Science Research Council with a grant to study the mechanical properties and fracture of invertebrate hard tissues, and by the Cocos Foundation. Professor S. A. Wainwright provided space and equipment for part of the work. We wish to thank A. J. Cain, D. Crenshaw, W. A. Newman, F. Vosburgh, and S. A. Wainwright for their helpful criticism.

SUMMARY

1. The shells of specimens of *Semibalanus balanoides* and *Balanus balanus* were loaded in compression. For shells of similar mass or pseudovolume (l × b × h), *B. balanus* was about four times as strong as *S. balanoides*.

2. The morphology of the shell plates and their junctions is described. The shell of *B. balanus* is mechanically superior to that of *S. balanoides* in its possession of a calcified basis, well developed radii and in its general workmanship.

3. Neither the microstructure nor the microhardness of the shell plates gives significant indications that differences in material properties could account for the differences in strength observed.

4. It is suggested that *S. balanoides* has a life history with a short life expectancy which makes it important to reproduce early in life and that, compared with *B. balanus*, neatness of shell construction and, hence, shell strength is sacrificed to speed in the process.

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COLLISELLA AUSTRODIGITALIS SP. NOV.: A SIBLING SPECIES OF LIMPET (ACMAEIDAE) DISCOVERED BY ELECTROPHORESIS

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Mayr (1963, p. 34) defines sibling species as "morphologically similar or identical natural populations that are reproductively isolated." During the past decade biochemical analyses have confirmed the differences and genetic isolation of numerous siblings that were originally recognized on the basis of breeding tests, cytological comparisons, or slight morphological differences. Included in such studies have been species of *Drosophila* (Hubby and Throckmorton, 1968; Prakash, 1969; Ayala, Mourão, Pérez-Salas, Richmond, and Dobzhansky, 1970), mammals (Johnson, Selander, Smith, and Kim, 1972), and seastars (Schopf and Murphy, 1973). In one instance biochemical data provided the first evidence that what had been considered a single species of sea cucumber was actually two morphologically similar species (Manwell and Baker, 1962; Manwell, 1966), and in the marine polychaete worm *Capitella capitata* (Fabricius, 1780) six sibling species have been distinguished on the basis of electrophoretic patterns of enzyme loci (Grassle and Grassle, 1976).

Limpets currently classified as *Collisella digitalis* (Rathke, 1833) on the basis of morphology range from the Aleutian Islands to southern Baja California (McLean, 1969). They are common throughout this range in the middle and upper intertidal zones, and are among the most eurytopic and phenotypically plastic of the eastern Pacific Acmaeidae. In the course of a geographic survey of two enzyme coding loci, two partially sympatric, reproductively isolated units were discovered within this taxon.

MATERIALS AND METHODS

Locations

Ten localities, covering a range of 1200 air kilometers, were sampled (Fig. 1). In July and August of 1977 samples were collected from the following sites in California: Corona del Mar State Beach (COR); Point Mugu (MUG); Gaviota State Beach (GAV); Jalama County Park, Santa Barbara County (JAL); Cayucos State Beach (CAY); the mouth of Mill Creek, 7 km south of Lucia (MIL); the Hopkins Marine Station, Pacific Grove (PAC); the mouth of Younger Lagoon, Santa Cruz (SAN); and the west side of Bodega Head, Sonoma County (BOD). Yachats State Park, Yachats, Oregon (YAC) was sampled in March, 1977.

Method of collection

Collisella digitalis exists in at least two ecotypic forms. One of these is found in beds of the stalked barnacle, *Pollicipes polymerus* (Sowerby, 1833), and the

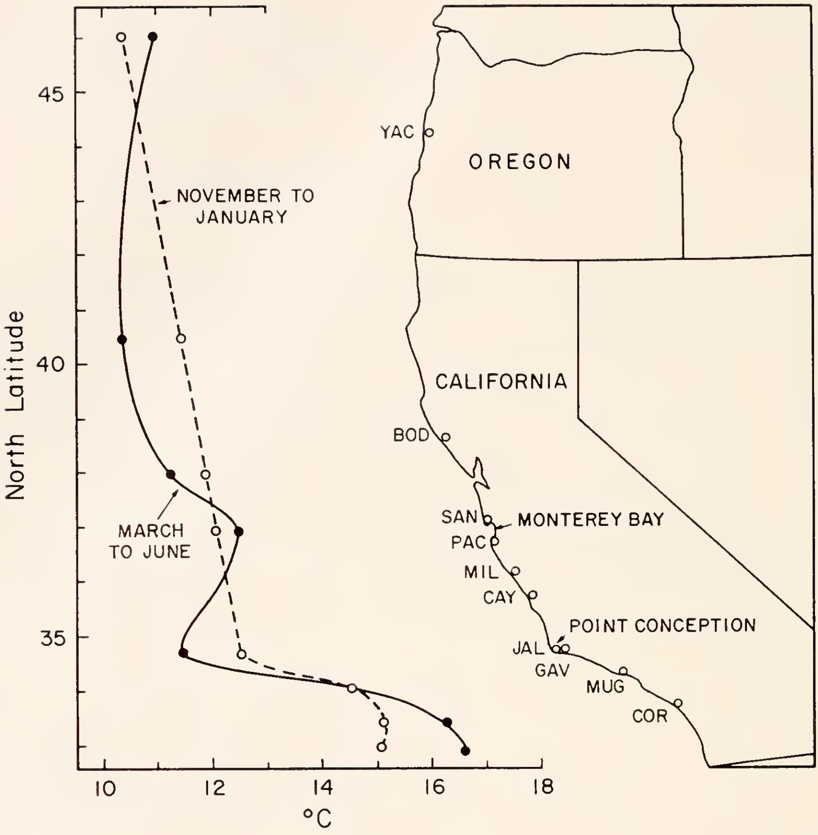
45
40
35
North Latitude
NOVEMBER TO JANUARY
MARCH TO JUNE
10
12
14
16
18
°C
OREGON
CALIFORNIA
YAC
BOD
SAN
MONTEREY BAY
PAC
MIL
CAY
POINT CONCEPTION
JAL
GAV
MUG
COR

FIGURE 1. Map of California and Oregon showing locations of sampling sites. Graph of mean March to June and November to January surface temperatures shows a thermal break at Point Conception and anomalous conditions near Monterey Bay (after Sverdrup, Johnson, and Fleming, 1942, p. 724).

other is found higher on intertidal rock surfaces. Giesel (1970) has suggested that phenotypic plasticity alone does not account for these morphs, but that predation pressure may select them from a continuum of shell types during each postlarval generation. Therefore, to assure as representative a sample of genotypes as possible, approximately equal numbers of specimens were collected at each site from stalked barnacle beds and from higher zones on the same rocks, except at YAC where the barnacle beds were not sampled.

Because it is difficult to identify small limpets to species, only specimens longer than 5 mm were collected. Otherwise, no conscious selection of any size class or shell pattern was made.

Limpets were brought alive to the laboratory and then kept at −76° C until electrophoretic analysis.

Electrophoresis

The digestive glands of individual snails were removed and homogenized in 1 ml polethylene BEEM capsules using the roughened ends of glass rods as pestles. The capsules were centrifuged at 4500 × *g* for 10 min and the supernatant absorbed onto 5 × 6 mm pieces of Whatman 3MM filter paper for insertion into starch gels. Gel composition, electrophoresis, and the leucine aminopeptidase (Lap) staining method have been described (Murphy, 1976). Phosphoglucose isomerase (Pgi) alloenzymes were visualized by staining for 5 to 10 min in a solution of 2 mg phenazine methosulfate, 7 mg NADP, 15 mg MTT, 20 mg fructose-6-phosphate, 30 units of glucose-6-phosphate dehydrogenase, and 160 mg $MgCl_2 \cdot 6H_2O$ in 70 ml 0.05 M Tris-HCl buffer, pH 8.0. Horse ferritin (Sigma) was used as a marker, and individuals of known genotypes served as standardizations of mobility variants between samples.

RESULTS

Lap polymorphisms

The zymograms reveal one major zone of Lap activity, and, assuming a correspondence between bands and alleles, a total of 10 codominant alleles were found at the locus coding for this zone. The Lap phenotypes of homozygotes are double bands about 4 mm apart, and those of heterozygotes are four bands, or three where the fast band of the slower allele has the same mobility as the slow band of the faster allele (Fig. 2). Allele abbreviations follow Ayala, Powell, Tracey, Mourão, and Pérez-Salas (1972). The most common allele

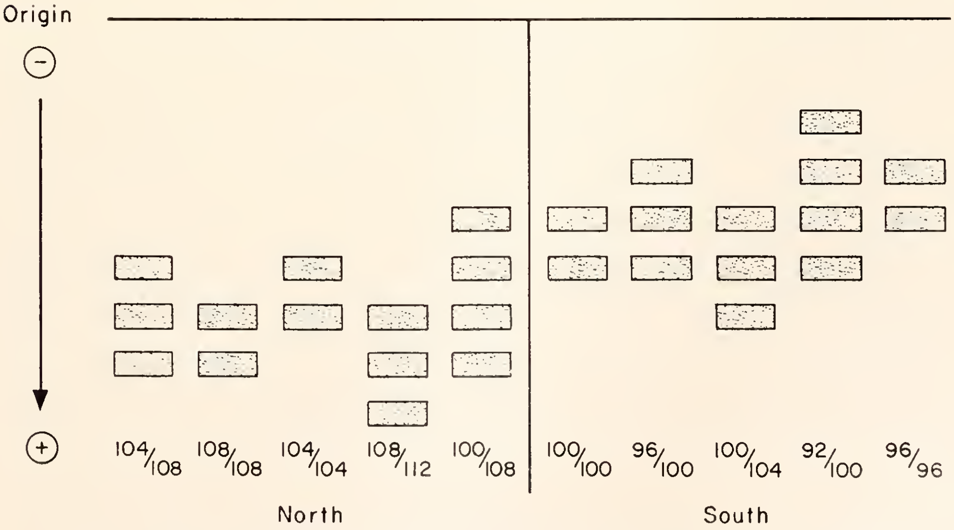

FIGURE 2. Banding patterns of the five most frequent Lap phenotypes in the "north" and "south" groups.

TABLE I

Observed genotypes, allele frequencies, and chi-square goodness of fit tests at the Lap locus.

	Localities									
	YAC	BOD	SAN	PAC	MIL	CAY	JAL	GAV	MUG	COR
Genotypes										
88/100	0	0	0	0	0	1	0	0	1	1
92/92	0	0	0	0	0	0	0	1	1	1
92/96	0	0	0	1	0	0	1	3	0	5
92/104	0	0	0	1	0	0	0	0	2	1
94/100	0	0	0	0	0	0	1	1	0	1
96/96	0	0	0	1	0	2	5	1	2	4
96/102	0	0	0	0	0	0	0	0	1	1
96/104	2	0	0	1	0	1	4	1	3	1
98/100	0	0	0	0	0	0	4	1	2	0
92/100	0	0	0	4	2	8	8	8	9	11
96/100	0	0	0	4	3	14	13	22	27	22
100/100	0	1	0	43	8	52	59	58	79	106
100/104	0	0	1	4	4	5	4	6	10	13
100/102	0	0	0	0	0	0	0	0	0	1
104/104	9	31	23	13	28	14	14	3	1	0
108/108	22	45	47	11	49	12	12	0	0	0
104/108	20	53	50	20	57	29	18	0	0	0
100/108	0	1	3	1	1	0	1	0	1	1
96/108	1	0	0	0	4	3	1	1	0	0
102/108	1	0	0	0	0	0	0	0	0	0
100/112	1	0	1	0	0	0	0	0	0	0
104/112	2	0	1	0	1	0	0	0	0	0
108/112	1	5	1	0	1	0	0	1	0	0
112/112	1	0	0	0	0	0	0	0	0	0
Accord with Hardy-Weinberg										
X^2	1.47	3.85	2.12	86.1	18.1	76.9	83.1	1.61	0.671	2.79
df	2	2	2	4	4	6	5	3	3	3
P	0.48	0.15	0.35	<0.001	<0.01	<0.001	<0.001	0.66	0.88	0.43

in the five southernmost samples is used as standard, Lap 100. The genetic hypothesis is that the double bands represent two polymeric enzymes that share a common polymorphic subunit as well as having different monomorphic subunits (Shaw, 1964).

Genotype frequencies for this Lap locus were determined for all ten localities, and their agreement with Hardy-Weinberg (H-W) expected frequency distributions was tested. Because of the large number of alleles at this locus, many genotypic classes contain few or no individuals, and classes with expected frequencies fewer than 5 were not included in chi-square goodness of fit tests. From Table I it is clear that the Lap genotype frequencies from localities between PAC and JAL inclusive do not conform to H-W expectations. It is also evident that, while they do not deviate significantly from H-W expectations, samples from sites north

TABLE II

Chi-square accord of Lap genotype frequencies after subdivision of samples PAC, MIL, CAY, JAL, and GAV into "north" and "south" subsamples.

	PAC		MIL		CAY		JAL		GAV	
	n	s	n	s	n	s	n	s	n	s
N	45	59	141	17	57	84	46	98	5	102
X^2	0.178	1.43	2.28	i*	0.157	0.621	1.48	4.02	i	0.500
df	2	3	2	—	2	2	2	3	—	3
P	0.92	0.70	0.32	—	0.92	0.67	0.48	0.26	—	0.92

* i = insufficient sample.

of PAC and samples from south of GAV have very few genotypes in common. There are four alleles in the MUG-COR samples and one in the YAC-BOD-SAN samples that are not shared by both groups. To test the hypothesis that two reproductively isolated groups had been sampled, the alleles of the YAC, BOD, and SAN samples were pooled into a "north" sample and those of MUG and COR into a "south" sample, and the probability of correctly assigning an individual to one of the samples thus formed was calcualted by the method of Ayala and Powell (1972). This probability is 98.8% which is very close to the 98.9% probability based on the overlap of the observed genotypes, and matches, to the nearest per cent, the arbitrary value of 99% defined as diagnostic by Ayala and Powell. By this method those Lap genotypes listed below 100/102 in Table I have a higher expectancy in the "north" group, and those listed above 104/104 a higher expectancy in the "south" group. If individuals in the samples between PAC and GAV inclusive are each assigned to a "north" or "south" subsample on on this basis, the significant ($P < 0.05$) deviations from H-W equilibrium are eliminated in every case, and at GAV, where less than 5% of the genotypes fall into the "north" subsample, the "south" subsample has a better fit to H-W expectations than does the undivided sample (Table II).

These results indicate two genetically isolated groups, each with some endemic alleles, that have very different frequencies for the Lap alleles that they share. North of PAC and south of GAV these groups are allopatric, or virtually allopatric, and between PAC and GAV inclusive they are sympatric. Assuming no hybridization in the zone of sympatry, the expected numbers of the Lap genotypes 96/104, 96/108, 100/104, and 100/108 were calculated for each of the sympatric samples. The totals for each of these genotypes are 3.6, 3.9, 24, and 1.6, respectively, while the observed numbers of these genotypes are 7, 9, 23, and 3. Since these four genotypes would account for the vast majority of "north" × "south" hybrids, there is little, if any, introgression evident in the 654 individuals making up the five sympatric samples.

Pgi polymorphism

The results of the Lap analyses are corroborated by the survey of the one major zone of Pgi activity that could be consistently scored. The locus coding for

TABLE III

Observed Pgi genotypes and chi-square accord with Hardy-Weinberg expectations for samples after subdivision into "north" and "south" subsamples on the basis of Lap genotypes. Parentheses enclose statistic for samples prior to subdivision.

	Localities														
	YAC	BOD	SAN	nPAC	sPAC	nMIL	sMIL	nCAY	sCAY	nJAL	sJAL	nGAV	sGAV	MUG	COR
Genotypes															
88/88	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
88/94	4	5	4	1	0	3	0	1	0	1	0	0	1	1	2
92/94	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
94/94	6	23	18	2	5	11	1	8	5	7	5	0	3	8	15
94/100	21	49	39	18	22	52	4	20	24	15	22	2	38	44	54
94/106	2	5	5	3	3	7	1	2	3	1	3	0	5	6	10
94/108	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
85/100	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
88/100	4	1	6	4	0	10	0	3	1	1	3	0	0	4	1
92/100	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0
100/100	20	42	38	14	24	45	8	22	39	12	38	3	43	62	75
100/106	3	10	13	2	5	12	2	0	10	2	1	0	9	9	11
100/108	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1
100/112	0	0	1	0	0	1	1	1	0	0	1	0	0	1	0
88/106	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
106/106	0	0	1	0	0	0	0	0	1	0	1	0	1	1	0
	Hardy-Weinberg accord														
X^2	0.103	2.15	3.07	0.657	0.014	1.88	0.218	0.868	0.105	0.357	0.274	i	2.62	0.502	4.69
				(0.763)*		(1.46)		(1.57)		(2.84)		(2.88)			
df	2	4	5	1	5	5	1	2	2	2	1	—	3	3	4
				(3)		(5)		(3)		(3)		(3)			
P	0.95	0.71	0.69	0.42	>0.99	0.87	0.64	0.65	0.95	0.84	0.60	—	0.45	0.92	0.32
				(0.86)		(0.92)		(0.67)		(0.42)		(0.41)			

this zone has a total of eight alleles, Pgi ^{86} to Pgi ^{112}, the allele used as standard being the most common in every sample. At this locus the electrophoretic phenotypes of homozygotes are single bands and those of heterozygotes are three bands, indicating a dimeric enzyme with a single polymorphic subunit.

The "north" and "south" groups established above on the basis of Lap genotypes differ much less with respect to the Pgi loci than they do for their Lap loci. None of the ten original samples differed significantly from H-W equilibrium, and division of the sympatric samples into "north" and "south" components results in only slightly better overall H-W agreement where Pgi genotypes are concerned (Table III). However, the mean frequencies of three of the four most common alleles, Pgi ^{88}, Pgi ^{94}, and Pgi ^{100}, in the "north" and "south" sympatric subsamples are statistically different ($P < 0.05$ by Student's *t*-test). The mean frequencies of these alleles in the sympatric subsamples are clearly similar to their frequencies in the corresponding northern and southern allopatric samples (Table IV). Only a linkage disequilibrium of unprecedented scale could account for this, were not two genetically isolated groups involved.

Status of the "north" and "south" groups

Acmaeid limpets have a planktonic larval stage lasting at least five days (Kessel, 1964; Proctor, 1968) and probably as long as three weeks or more if a suitable settling site is not available (Karp, 1970). Such species should have homogeneous gene pools with similar allelic frequencies over long distances, if not over an entire species range (Gooch, 1975; Soulé, 1976). Therefore the presence, in a single species, of a cline of Lap allele frequencies of the magnitude found here between the northern and southern ends of the range sampled would not be expected.

The larger than expected numbers of Lap genotypes 96/104 at JAL, 96/108 at MIL and CAY, and 100/108 at SAN may represent hybrids between the "north"

TABLE IV

Comparison of Pgi 88, 94, 100, and 106 frequencies for "north" (n) and "south" (s) samples. Parentheses enclose statistics comparing means of n and s subsamples from PAC, MIL, CAY, JAL, and GAV.

	88		94		100		106	
	n	s	n	s	n	s	n	s
PAC	0.078	0	0.289	0.297	0.578	0.636	0.056	0.068
MIL	0.046	0	0.291	0.206	0.592	0.676	0.067	0.088
CAY	0.036	0.012	0.342	0.220	0.596	0.673	0.018	0.095
JAL	0.050	0.020	0.388	0.230	0.525	0.691	0.038	0.039
GAV	i	0.005	i	0.250	i	0.657	i	0.078
$\bar{X}$	0.053	0.0074	0.328	0.241	0.573	0.667	0.043	0.074
t	(5.00)		(3.17)		(5.25)		(1.98)	
P	(<0.01)		(<0.02)		(<0.01)		(<0.05)	
Mean of YAC, BOD, and SAN	0.043		0.347		0.546		0.059	
Mean of MUG and COR		0.014		0.265		0.652		0.062

and "south" groups. However, with respect to taxonomy, the existence of hybrids is not crucial. Dobzhansky, Ayala, Stebbins, and Valentine (1977, p. 171) define *species* as "Mendelian populations, or arrays of Mendelian populations, between which the gene exchange is limited or prevented by reproductive isolating mechanisms." The small number of possible hybrids and the differences in allele frequencies at the loci studied, despite the groups' approximately 350 km zone of sympatry, clearly indicate a sufficient degree of reproductive isolation to qualify these groups for species status.

All samples, even after subdivision into "north" and "south" subsamples, have deviations from H-W equilibrium that, although they are not statistically significant at the sample sizes employed, reflect an excess of homozygotes. Several other authors have noted similar H-W deviations in marine invertebrates, including several species of mussels (Koehn and Mitton, 1972; Koehn, Turano, and Mitton, 1973; Tracey, Bellet, and Gravem, 1975; Koehn, Milkman, and Mitton, 1976), a lobster (Tracey, Nelson, Hedgecock, Shleser, and Pressick, 1975), a phoronid (Ayala, Valentine, Barr, and Zumwalt, 1974), and a population of *Collisella digitalis* from Bodega Head (Gresham and Tracey, 1975). This is best explained by strong microhabitat selection, proportionally greater within than between microhabit mating, and subsequent mixing of the resulting larvae (see Mitton and Koehn, 1973; Tracey, Bellet, and Gravem, 1975; Tracey, Nelson, Hedgecock, Shleser, and Pressick, 1975; Koehn *et al.,* 1976 for similar explanations). High fecundity organisms such as these should easily afford the cost of such selection pressure (Williams, 1975). However, Makela and Richardson (1977), ignoring the probable selection pressure stemming from competition, argue that the presence of two or more sibling species is an equally good explanation of excess homozygosity that involves no selective cost. To account for the residual homozygote excess encountered here, their explanation requires that both the "north" and "south" groups be composed of more than one species with similar allelic frequencies and identical range end-points. The assumptions required for this hypothesis seem to be too stringent to warrant its consideration.

Taxonomy

The type locality for *Collisella digitalis* is Sitka, Alaska (Rathke, 1833). Therefore, the "south" group differentiated here is the unnamed species. I propose it be named *Collisella austrodigitalis* Murphy, sp. nov. A holotype and paratypes, all from Corona del Mar, California (33°35′ N, 117°52′ W) and voucher specimens of *Collisella austrodigitalis* and *C. digitalis* from sites where the species are sympatric, have been deposited with the California Academy of Sciences, San Francisco. Paratypes have been deposited with the United States National Museum of Natural History, Washington, D. C.

The holotype was collected from the high intertidal rock surface. A core of its tissue was removed with a hollow needle and its genotype, for the two loci studied, was determined to be homozygous Lap ^{100} and homozygous Pgi ^{100}.

Morphological differences

The morphological similarity of these species is attested to by the fact that "*Collisella digitalis*" has been a frequent subject of taxonomic and ecological studies, yet no one has suggested that populations from northern and southern California belong to different taxa. Eurytopic acmaeids such as these exhibit striking shell variation in response to different ecological conditions (Test, 1945, 1946) that tends to mask differences between similar species. After determining species electrophoretically, examination of shells from sites where these species co-occur reveals some subtle interspecific differences. The shell interior tends to be more darkly shaded and the interior margin less often tesselated in *Collisella austrodigitalis,* while the shell exterior of *C. digitalis* has a more mottled pattern. Due to the variation mentioned above, there is much interspecific overlap with respect to these characters. Shell sculpture appears to be a more dependable diagnostic feature. *C. digitalis* tends to have broad, rounded ribs with convex intercostals, while the tendency of *C. austrodigitalis* is to ribs with square cross sections and flat intercostals (see Fig. 3). The spines on the ribs of *C. austrodigitalis* give them a beaded appearance. However, the sculpture on either species may be obscured by erosion or other phenotypic responses to the environment.

The radulae of these species are very similar. McLean (1966) studied "*C. digitalis*" radulae from both north of San Francisco and south of Point Conception without noting important differences. Fritchman (1960) found that collections of "*C. digitalis*" from north and south of Point Conception differed significantly with respect to two of the six radular characters he studied. The diagnostic value of his characters would not be great, however, since large numbers of northern and southern individuals shared the same character states.

Distribution

Temperature is generally considered to be one of the primary factors controlling the distribution of marine invertebrates (Dunbar, 1963; Kinne, 1970). Point Conception is the approximate southern range end-point of *Collisella digitalis,* and Monterey Bay the approximate northern limit of *C. austrodigitalis.* Both of these geographic features are well known for their peculiar hydrographic regimes and biogeographic significance. Point Conception is the recognized boundary between the warm temperate, Californian, and temperate, Oregonian, molluscan provinces. It is the range end-point for several hundred species of either province (Valentine, 1966). The dramatic thermal break at Point Conception (Fig. 1) is presumed to account for its biogeographic importance. The sites JAL and GAV are only 30 km apart, one on either side of Point Conception, and GAV is less than 2 km farther south than JAL, yet, there is a striking difference in numbers of *Collisella digitalis* at these sites (Table I). The comparative reduction of this more northern of the two species at GAV correlates with the abrupt thermal change. Monterey Bay is generally warmer than waters to both the north and south, and harbors many southern thermophiles that end their ranges there (Valentine, 1966). The greater frequency of *C. austrodigitalis* at PAC than at MIL, even though PAC is farther north, may be a response to the

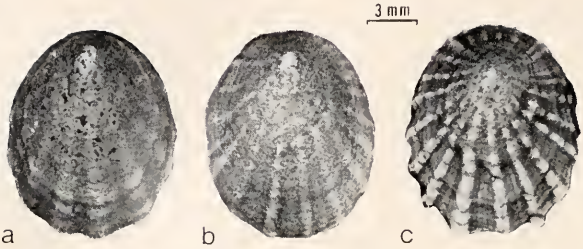

Figure 3. Shells of (a) *Collisella digitalis* from MIL; (b) *C. austrodigitalis* from MIL; (c) *C. austrodigitalis* from COR with sculpture similar to that of the holotype. Shells have been coated to accentuate sculpture.

higher temperatures near the bay. Addicott (1966) presents the possibility that unusually warm episodes of the annual Davidson Current period off central California (Bolin and Abbott, 1963) may result in blooms of southern species with planktonic larvae in this area. Recent hydrographic data (Lasley, 1977) indicate that such a warming cycle is in progress. Therefore, numbers of *C. austrodigitalis* in the vicinity of Monterey Bay may, at present, be higher than usual. Allelic frequencies previously reported for *C. digitalis* (Murphy, 1976) are in error because specimens of *C. austrodigitalis* were unwittingly included in the samples from the vicinity of Monterey Bay.

Discussion

Allopatric speciation seems to be the only possible mode of origin that can explain siblings such as *Collisella austrodigitalis* and *C. digitalis* that are restricted to the same habitats in the intertidal zone. However, the barriers that might have served to divide spatially the original species are not obvious. One possible chain of events, involving any of the late Cenozoic world wide climatic fluctuations is as follows. During warming trends warm-water species extend their ranges into higher latitudes, and during cooling intervals, as the polar-equatorial temperature gradient increases, they contract their ranges equatorward (Valentine, 1973). During episodes of range contraction populations might become isolated in suitably warm, higher latitude embayments (Addicott, 1966 considered this as a possible explanation for the presence of a warm-water, late Pleistocene fossil assemblage in northern California). Should this isolation last long enough, speciation would occur. Golikov (1973) predicts that cold adaptation in such an isolated population would accelerate speciation. Then during a subsequent warming interval sympatry between the new, but still morphologically and ecologically similar, species might be reestablished. If this chain of events does explain the evolution of *Collisella austrodigitalis* and *C. digitalis*, then the southern *C. austrodigitalis* is the older species, and the isolation and cold adaptation, if not speciation, of *C. digitalis* was complete by the late Pleistocene since "*C. digitalis*" is present in a cool temperate, Aleutian, fossil assemblage of that date near Bandon, Oregon (Zullo, 1969). Investigation of other molluscan species with ranges overlapping two or more molluscan provinces may reveal other examples of such siblings.

Speculation as to the isolating mechanisms that prevent introgression of these siblings is difficult because of the paucity of information on acmaeid reproduction. Dissynchronization of spawning is a possibility, but Fritchman (1961), while observing some seasonality of spawning in *C. digitalis* in the vicinity of San Francisco, found that in favorable habitats some spawning occurred year around. Thus, it seems unlikely that this mechanism could account for the degree of isolation observed unless there is increased periodicity of spawning near the range end, perhaps due to brief annual exposure to critical temperatures there. Of the other possible mechanisms that could operate, gametic isolation seems unlikely to have evolved except in response to zygotic mortality or hybrid inviability, but either of these latter mechanisms is a sufficient, and perhaps most reasonable, explanation for the lack of introgression observed.

Whether or not introgression is entirely absent cannot be determined from the present data. Even *C. digitalis* from YAC and *C. austrodigitalis* from COR, the terminal sites of the range studied, have some Lap alleles in common, and, therefore, Lap genotypes that are identical to those that should be most frequent among hybrids. With the criterion used for distinguishing the species, and assuming no hybridization, the Lap genotypes 96/104 and 96/108, although rare, are approximately twice as frequent in the zone of sympatry as expected. This fact in conjunction with the three 100/108 and the one 100/112 Lap genotypes at SAN, which is well within the dispersal distance of the zone of sympatry, suggests a small amount of hybridization. However, a definitive study of this point will have to employ larger samples or additional loci. By including more loci, especially if other diagnostic loci can be found, not only can the frequency of hybrids be determined, but, if hybrids do exist, the extent to which F 1 hybrids backcross to either parental species may be estimated using the logic Hall and Selander (1973) applied to their study of hybridizing lizards.

I am grateful to Kathleen D. Murphy for her technical assistance, and to Katherine A. Coffaro and Ralph T. Hinegardner for their suggestions concerning the manuscript. This research was partially supported by NSF grant DES74–19476 to John S. Pearse.

SUMMARY

A geographic survey of protein polymorphism in the intertidal limpet *Collisella digitalis* reveals that populations from northern California and Oregon have allelic frequencies at a leucine aminopeptidase locus that are sufficiently different from populations in southern California that individuals can be correctly assigned to their geographic entity with a 98.8% probability on the basis of their genotype. These two groups also have different allelic frequencies at a phosphoglucose isomerase locus, but only slight morphological differences between the groups are evident.

Genotype frequency analysis shows that on the central California coast between Monterey Bay and Point Conception both the northern and southern groups are

present, but not interbreeding. Thus, these groups are sibling species. The southern species is herein named *Collisella austrodigitalis* sp. nov.

These species are thought to have originated by allopatric speciation, perhaps following the spatial isolation of a population of *C. austrodigitalis* during an equatorward contraction of that species' range in response to one of the late Cenozoic world wide cooling trends.

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REPRODUCTION IN TWO SYMPATRIC SPECIES OF *MACOMA* (BIVALVIA)

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The phenomenon of sexual differentiation in molluscs has been well reviewed (Coe, 1936, 1940, 1943a). Although a great deal of work has been done on gonadal development in Lamellibranchs, most of it concentrates in seven families: Pectinidae (Sastry, 1963); Teredinidae (Coe, 1943b); Veneridae (Ansell, 1961); Ostreidae (Dinamani, 1974); Mytilidae (Bartlett, 1972); Myacidae (Pfitzenmayer, 1962); and Mactridae (Machell and DeMartini, 1971). The only member of the Tellinidae studied to date is *Macoma balthica* (Lammens, 1967; Caddy, 1967).

Macoma secta (Conrad, 1837) and *M. nasuta* (Conrad, 1837) are geographically sympatric species of the bivalve family Tellinidae. *M. secta* occurs in intertidal sand flats to sediment depths of 60 cm from Vancouver Island, British Columbia, to Bahia Magdalena, Baja California; while *M. nasuta* occurs in muddier substrates intertidally to sediment depths of 40 cm from Kodiak Island, Alaska, to Cabo San Lucas, Baja California (Coan, 1971). Ricketts, Calvin, and Hedgpeth (1969) list *M. secta* as occurring in the Middle Intertidal Zone and *M. nasuta* in the Low Tide Horizon. They are found in decreasing numbers to 25 fathoms (Abbott, 1954). *M. secta* and *M. nasuta* are the characteristic species of the *Macoma* community on the west coast of North America. Thorson (1966) had identified five *Macoma* communities worldwide.

In the present study the seasonal gonadal changes and sex ratios of *Macoma secta* and *M. nasuta* are reported as well as the physical and biotic factors which may influence them. This includes complete qualitative descriptions of the stages of gametogenesis. The reproduction of these species is compared and the reproductive advantage each species has to its particular milieu is examined. Of particular interest is the comparison of the two populations studied, viewing the effectiveness of reproductive barriers. There exists no physical barrier between these sympatric populations. Therefore, crossing must be prevented through environmental and/or physiological barriers.

MATERIALS AND METHODS

Macoma secta and *M. nasuta* occur sympatrically on Lawson's Flat, a sandy intertidal area, located 0.8 mile from the mouth of Tomales Bay, California. From March 2, 1974, to March 31, 1975, fifteen clams of each species were collected bi-weekly at each spring low tide. An effort was made to select clams from their respective tidal midranges in order to avoid animals living in stress. The midranges for *M. secta* and *M. nasuta* are 45 and 15 cm above mean low water, respectively.

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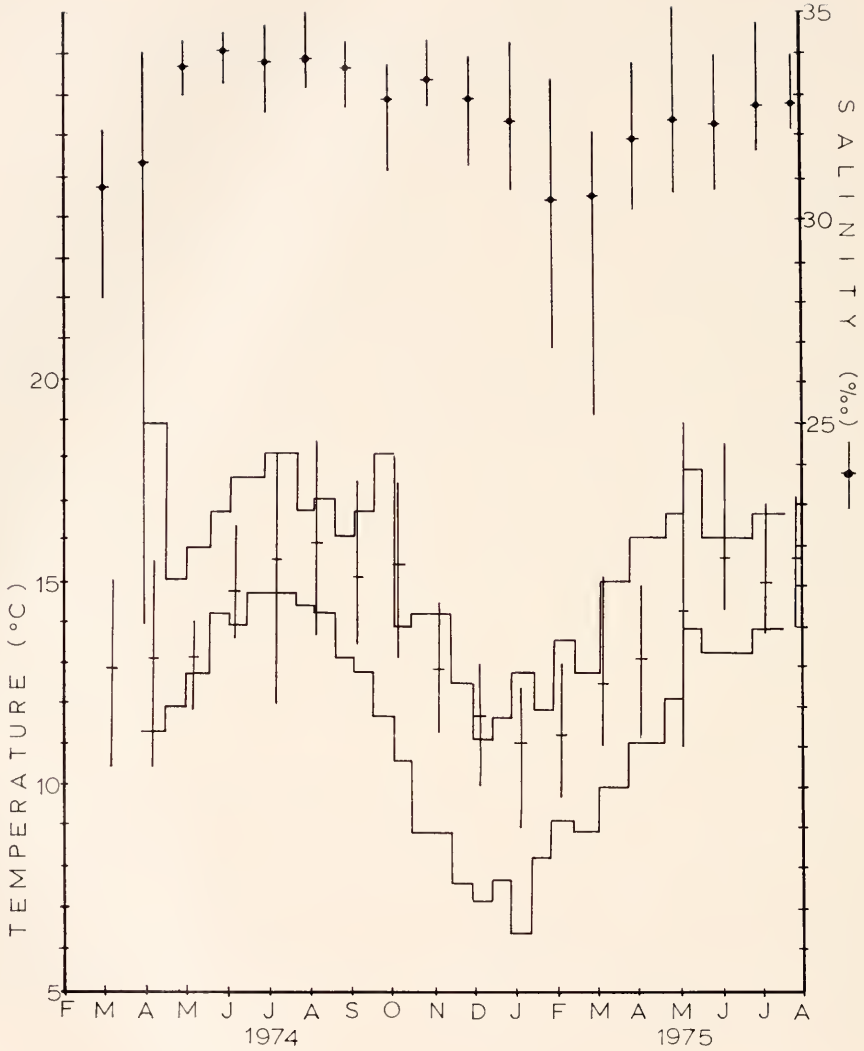

Figure 1. Pacific Marine Station weather station data: sea water temperatures and salinities; monthly means and ranges. Superimposed are interstitial maximum-minimum temperatures, taken at 40 cm depth at +60 cm tidal height.

A maximum-minimum thermometer was placed in the sediment to a depth of 40 cm at a tidal height of 60 cm and read bi-weekly. Sea water temperature and salinity were recorded daily at Pacific Marine Station one mile from the Lawson's flat site and are presented in Figure 1.

After collection, the clams were placed in flowing sea water pumped directly from the Pacific Ocean; consequently, the possibility of thermal shock causing gamete release was minimal. They were kept for one day to cleanse the alimentary tract of most ingested sand. Because of the weakly-developed adductor muscles in *M. secta,* the animals tend to gape and become moribund when left out of the sediment. As a counter measure, rubber bands were used to hold the valves together. This measure was not necessary for *M. nasuta* which has strong adductor muscles. The clams were assigned a number and measured for length and width using vernier calipers. The presence of commensals was noted.

The visceral mass was cut on a transverse axis at a point below the attachment of gills and palps. The lower (ventral) portion was preserved in Bouin's fixative for 24 hours. A 4 mm subsample was dissected and processed by standard histological technique. The tissue was dehydrated with isopropanol, cleared with toluene and embedded with 56 to 58° C paraffin. Sections were cut at 8 to 10 μ, stained progressively with a modification of Harris' hematoxylin (Galigher and Kozloff, 1971, p. 352) and counterstained with Eosin Y.

In studying the seasonal gametogenic cycle, only adults were used. Mature clams with fully differentiated gonads were encountered at sizes above 3.0 cm for *M. secta* and 2.4 cm for *M. nasuta.* Primordial gonadal development was not studied for protandry. Caddy (1967) illustrated the external appearance of the visceral mass of *Macoma balthica* as it passed through the gametogenic cycle. Descriptions for *M. secta* and *M. nasuta* are identical to *M. balthica;* therefore, they are not repeated.

Histological examination of the gonads in *Macoma* allowed the seasonal gonadal cycle to be broken into six stages: inactive; early active; late active; ripe; partially spawned; and spent. This sequence is modified from Ropes and Stickney (1965) for *Mya arenaria.*

RESULTS

Stages of gonadal development for Macoma *and their criteria*

In keeping with Raven's (1961) classification of oogenesis, the term *follicle* is not used here for the basic gonadal developmental unit because *follicular* egg development implies a completely different process from *solitary,* that found in most bivalves. The term *alveolus* is used as described in Ropes and Stickney (1965).

Inactive. The main criterion for this stage is the presence of the contracted alveolus. In *M. nasuta* the vacuolated follicle cells completely fill the alveolus. Sex can usually be determined in males by the presence of residual spermatozoans. It appears the unshed spermatozoa will remain viable over the winter to be shed with the next year's sperm. With females, sex determination is more difficult as residual ova are rarely found. Females in this stage are usually identified by the presence of many noncellular spherical inclusions in the alveoli, which are probably nutritive in function and albuminous in composition. Similar inclusions were described for *Mya arenaria* (Coe and Turner, 1938). Phagocytes are extremely rare in this and other stages. The alveoli have a highly organized appearance in this species and are round and firm.

The general appearance of the gonads of *M. secta* at this stage is characterized by flaccid compressed alveoli in a matrix of disorganized and pycnotic connective tissue in a state of phagocytosis. The development of vacuolated follicle cells is varied, ranging from none to a thin one cell layer covering the alveolar wall.

Early active. Following the inactive period, gametogenesis is initiated in the early active stage. This is the earliest stage in the next cycle in which the clams can be sexed.

In females of *M. nasuta,* meiosis has produced primary and secondary oogonia and young oocytes have begun to grow out from the alveolar wall. In males, primary and secondary spermatogonia are rapidly being produced, starting to fill in the lumen. Some spermatogonia will be seen throughout the alveolus because they are able to migrate within the matrix of follicle cells. The smaller darkly stained spermatids are usually present towards the center of the lumina. The gonads are more organized as the alveoli anastomize into spaces between muscles and digestive diverticula.

The description of early active for *M. secta* is similar to *M. nasuta* except that phagocytosis is active in *M. secta,* especially in males, resorbing unshed gametes. Also, the male meiotic products of *M. secta* are more closely associated with the alveolar wall, with advanced stages extending into the lumen. This is a result of the poorly developed follicle cells.

Late active. This stage is characterized by a rapid proliferation of gametes. Spermatozoa of both species form streams with their tails extending into the lumen. The arbitrary qualitative maximum limit of spermatozoa present is seventy per cent of the meiotic cells present. In females of both species, oocytes begin to elongate, extending into the lumen on thick stalks. Amphinuclei are evident as they move to the distal end of the oocytes. Very few eggs are mature and most still adhere to the alveolar wall by at least a slender stalk. Young oocytes are present on the wall.

There are a few note worthy differences between species. Male meiotic products are arranged differently. In *M. secta* advancing stages are found increasingly closer to the lumen center. In *M. nasuta* the products are forced into crevasses between the follicle cells resulting in a patchy appearance. Also, the follicle cells of *M. nasuta* decrease in number as gametes are produced. Lastly, the female spherical bodies of *M. nasuta* continually shrink, both in size and number.

Ripe. All clams in this stage are physiologically prepared to spawn. For males of both species, most of the lumen is filled with spermatozoa; qualitatively, the arbitrary minimum of spermatozoa is 70% of all alveolar cells. The sperm form long streams extending into the lumen, with their tails directed toward the center. The first stages of gametogenesis become less evident. For females, almost every oocyte is mature and oval shaped, free in the lumen. A few are still attached by slender stalks to the alveolus wall. Young oocytes are absent from the alveolar walls. Unspawned oocytes of *M. secta* average 51.7 μ with a range of 46 to 57 μ, while oocytes of *M. nasuta* ranged from 48 to 57 μ with a mean of 52.6 μ.

Partially spawned. In this stage there are obvious gaps in the lumina indicating that some gametes have been shed. Most remaining gametes are mature. In both sexes, there is little or no early gametogenic activity.

This ephemeral stage in *M. nasuta* is rarely witnessed. Follicle cells begin to grow on the basal membrane of the alveolus. Individuals appear to spawn in a

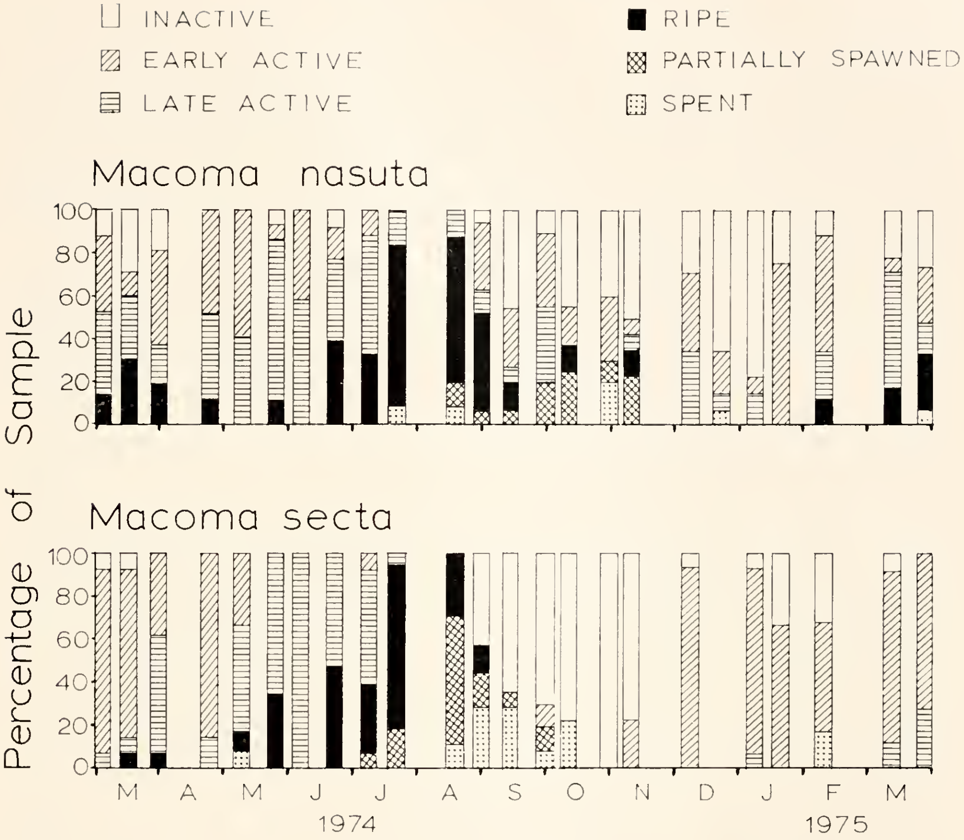

FIGURE 2. Comparison of population gonadal state of *Macoma nasuta* with *M. secta.*

very short period of time. Simultaneously, the follicle cells grow rapidly to fill the empty lumen. Frequently the new year's primary gonia begin growing within the follicle cell matrix. The small spherical cell inclusions of the females begin to appear.

Alveoli of *M. secta* initiate neither follicle cell production nor gametogenesis. Phagocytosis is sometimes in evidence.

Spent. This stage in *M. nasuta* is also shortly lived. A few residual gametes are left and follicle cells fill the alveoli. The females' spherical inclusions have grown in size and in numbers. The alveoli of *M. nasuta* remain organized and make a smooth, quick transition to the inactive state.

In *M. secta,* the alveoli have a shrunken, compressed appearance. Only a few residual gametes are left to identify sex. Phagocytosis is evident inside and outside of the alveoli, consuming pycnotic cells. Tissue is in extremely poor condition. This is a regressive stage with no cell buildup.

Gametogenic cycle

Macoma secta. Gametogenesis in *M. secta* began in November as the population entered the early active phase (Fig. 2). Gonadal development throughout the winter months was, however, very slow. Most clams remained in the early active stage until April. During this time the minimum sediment temperatures were consistently below 10° C, and the lowest seawater temperature averaged 11.0° C (Fig. 1). Besides the thermal stress, the rainy season during the winter months caused a drop in the average salinity.

As temperatures increased in April and the rainy season ended, the rate of gonad development increased and the late active phase was entered. This period was shorter and lasted approximately through June. Great numbers of sperm were found in the testes, which appeared to be fully viable, whereas very few mature oocytes were encountered; most eggs were still connected to the basal membrane of the alveolus.

Ripe clams were encountered primarily in July and early August. The definition of ripe given here may be more restrictive than previous works. However, it is to be shown that spawning occurs very rapidly for an individual, on the order of two to three weeks maximum. Those individuals considered ripe must necessarily be near completion of gametogenesis. This narrow point in the cycle was reached at the time of the highest yearly temperatures (monthly mean) which for both the seawater and sediment temperatures approximated 15.5° C.

Most spawning occurred in August. There may have been a temperature stimulus for this since average seawater and sediment temperatures began to drop toward fall readings. The discharge of gametes was accomplished quickly for the population as a whole and the spent stage was entered in late August (Fig. 2). The gonads were in a disrupted state at that time. While temperatures were still high enough for the clams to effect repair, the inactive state was very quickly entered in September. The spent stage was by far the shortest. Follicle cells began to fill the alveoli. The gonads started to reorganize, building up reserves for the early active period which started again in November.

Macoma nasuta. Although the same six stages well describe the gametogenic series in *M. nasuta*, their synchrony with time differed greatly from *M. secta*. In general, the partially spawned and spent stages were rarely encountered, owing to their extremely short duration. The remaining four stages were present in some numbers in nearly every month (Fig. 2). Exceptions were few; no clams were found in the inactive stage in July and August, early active stage in August or ripe stage in December and January. Consequently the gametogenic pattern was somewhat obscured.

Despite these problems, there did appear to be a seasonal pattern. The spring spawning schedule was as follows. From a maximum of clams in the inactive stage in December and January, the early active stage was entered in January and February. In March they rapidly entered the late active stage. Late in that month and in April, they apparently spawned as the number of ripe individuals in the samples decreased. No partially spawned clams were noticed in this series, but that was not surprising. The seemingly premature presence of spent individuals in July and August may be explained by this spring spawning, which was very slight and, based on field observation of recruitment, may not often be successful (Rae, 1975).

The main gametogenic series occurred in the fall spawning schedule. The inactive period was left in March and gametogenesis was initiated in April and May. The clams continued gamete buildup into May and June, finally ripening in July and August. The population appeared to spawn over a wide period of time from August to November, as evidenced by the occurrence of the partially spawned state. Thereafter, the spent stage was briefly passed through and the clams became inactive. As will also be shown later, the partially spawned and spent stages were extremely short in duration and are presented here basically for continuity. Ripe individuals essentially passed to the inactive stage. Individuals probably spawned all at once, as the apparent brevity of the spawning stage indicates, however as a population, this was accomplished slowly.

It is possible that many clams stretch their gametogenic period over the spring and fall schedules. Surely there are many clams that do not fit either schedule. Also it is suspected that some spring spawners build up gametes early and simply remain ripe for a long period of time, spawning in the fall.

The correlation with temperature noticed in *M. secta* was not readily noticed for *M. nasuta*. *M. nasuta* may have a rather plastic endogenous reproductive rhythm that temperature controls in some complex way. The average temperatures found at the time of spring and fall spawning were approximately the same (about 14° C). Possibly this optimized larval survival because of the temperature itself or because of a timing factor.

Comparative gametogenesis

The results indicate distinct variation in gametogenic cycles in the genus *Macoma*. In order to view the population as a whole, the stages inactive through spent were assigned numbers 1 through 6 with a new cycle starting with 7 for inactive and continuing likewise. In this way a nonparametic mean could be assessed for the population gonadal state on each collection date (Fig. 3).

Generally *M. nasuta* developed gametes earlier in the winter than *M. secta*. However, *M. secta* constantly advanced in the cycle while *M. nasuta* remained in the active stage longer. Spawning was rapid for *M. secta* in mid-August whereas the *M. nasuta* population was slower, having spawned from mid-August through November. The graph was split during this period for *M. nasuta* because the distribution of stages was bimodal. This was because of the rapid change from the ripe to the inactive or early active state for individuals. One mean for the samples in this period would falsely indicate the nature of the population's slow progression through the spawning stages. As the mean's ranges indicate, the *M. nasuta* population was more variable as to gonadal state.

Although the two species proceeded through the gamete buildup with approximately the same timing, spawning did not appear to overlap. *M. secta* spawned in August whereas *M. nasuta* appeared to spawn slightly in May and again from September through November. This minimized the loss of gametes to cross fertilization, a distinct possibility for sympatric species.

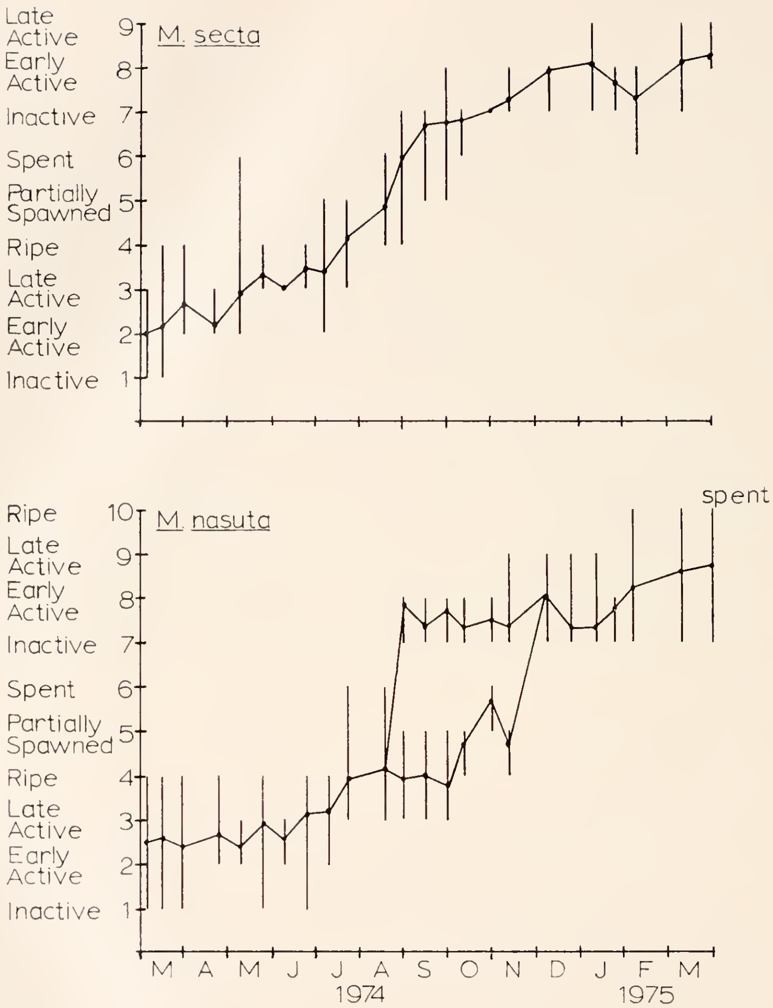

FIGURE 3. Gonadal development of *Macoma secta* and *M. nasuta* populations. Means and ranges are presented for each collection date. The ordinate represents advancing stages of gametogenesis.

Sex ratios

In this study, gonadal sections from a total of 344 individuals of *M. secta* and 336 of *M. nasuta* were examined. For *M. secta* the total sex ratio was 149 males: 138 females: 54 indeterminate. For *M. nasuta* the ratio was 150 males: 156 females: 19 indeterminate. It is difficult to determine accurate sex ratios during the inactive phase, because males often retain sperm and females rarely retain eggs. Thus, observed ratios would be biased in favor of males with many females being classed as indeterminate. To counter this bias, the sex ratio was determined by adding together only those samples wherein all specimens were sexed. Chi-square tests were performed against a 1 : 1 sex ratio. Nonsignificance was found in both cases (*M. secta* $X^2 = 0.627$, $0.5 > P > 0.1$; *M. nasuta* $X^2 = 1.10$, $0.5 > P > 0.1$). The null hypotheses of 1 : 1 sex ratios were accepted.

It is not certain whether there is sex reversal or not in *M. secta* due to the impossibility of sexing during the inactive stage. However, most of the *M. nasuta* individuals are sexable throughout that period, and no indications of sex reversal were found.

Commensals and parasites

The presence of commensals may have an effect on reproduction and certainly the presence of parasites can have a devastating effect on reproduction and population stability (Obrebski, 1975). All clams of both species were examined for commensals and parasites, both those attached to the mantle and visceral mass, and those which may have embedded in the gonad or digestive diverticula, common attack points.

Both species were found to be remarkably free from both types of pests. No commensals or parasites were found on *M. nasuta.* One specimen of *M. secta* was completely castrated by the presence of a digenetic trematode. Three specimens of *M. secta* had commensals attached to the mantle near the ctenidia. They were identified as the nemertean, *Malacobdella grossa.* Examination of the infected clams revealed completely normal gonadal development. Addicott (1952) reported the incidence of *M. grossa* commensalizing *M. secta* in Elkhorn Slough as 15.4% and for *M. nasuta* 6.0%.

Spawning

On different occasions in August, 1974, spawning of ripe animals was attempted. Various thermal and chemical methods were tried, including heat shock, cold shock, and slowly increasing water temperature in conjunction with introduction of sperm and/or egg suspension. All these attempts proved ineffectual in spawning either species. Caddy (1967) also had poor results with *M. balthica.* After repeated attempts, he succeeded in inducing only a few clams to spawn on one occasion.

Discussion

No hermaphrodites were found for either species, providing evidence that adults of both species are probably gonochoristic. Coe (1945) mentioned that out of 10,000 bivalve species, only 400 are known hermaphrodites. It is unknown whether either species is protandrous; common for gonochoristic species. Caddy (1967) indicated a strong possibility for this in *M. balthica,* while Lammens (1967) doubted protandry for the same species.

Typically in bivalves, connective tissue is found around the alveoli as well as the follicle cells within the alveoli. This has been demonstrated in *Crassostrea virginica* (Loosanoff, 1942, 1965) and *Ostrea edulis* (Loosanoff, 1962). Since this tissue disappears with the growth of alveoli, Loosanoff (1942) attributed a nutritive function to it. However, this connective tissue is never found in either *M. secta* or *M. nasuta.* Lammens (1967) reported this also for *M. balthica.* He tested the inner follicle cells histochemically with sudan black B and obtained positive results indicating a probable nutritive function. Coe and Turner (1938) also attributed a nutritive function to these cells in *Mya arenaria* which has a similar connective tissue structure as *Macoma.* Follicle cells in *M. secta* and *M. nasuta* also seem to play an important role in alveolar nutrition.

There is a great difference in the post-spawning state between *M. secta* and *M. nasuta.* In *M. secta* as in *Mytilus edulis* (Chipperfield, 1953), the gonad disappears, only disorganized cells being left. However, for *M. nasuta,* as with *Cyprina islandica* (Loosanoff, 1953) and *Venus striatula* (Ansell, 1961), the alveoli retain their shape and fill up with the vacuolated follicle cells.

Both *M. secta* and *M. nasuta* initiated gametogenesis in late fall, but both also entered a period of dormancy during the winter months. Examination of Figure 3 suggests some gonadal regression was possible during this time for both species. The timing of this possible mid-winter resorption did correspond to the most stressful period of the year, January, when heavy Pacific storms brought much rain and cold weather. As demonstrated by Loosanoff and Davis (1952) *Crassostrea virginica* was unable to metabolize glycogen below 10° C. Minimum sediment temperatures were below this temperature during the winter. This may in part explain the observed dormancy. The range of salinities encountered (Fig. 1) increased, which produced a high environmental variability and uncertainty in osmotic stress for the clams. By comparison, the closely related *M. balthica* undergoes active gametogenesis in the winter, from September through February, in the English Channel with no dormancy whatever (Caddy, 1967).

The indications are that temperatures exogenously control spawning for bivalves. The relationship is far from simple. Temperature may optimize a species adult physiological development or optimize its larval survival as a timing mechanism. Loosanoff and Davis (1952) showed that a temperature of 15° C must be reached in order for *Crassostrea virginica* to spawn. However *C. virginica* has been shown to have physiological races which require varying temperatures above 15° C to accomplish gametogenesis and spawning (Loosanoff and Nomejko, 1951; Loosanoff, 1969). This subject is well reviewed by Giese (1959).

There are many variations in the timing of gametogenesis in bivalves. For populations of *M. balthica,* in the Netherlands (Lammens, 1967) and in the Thames Estuary, England (Caddy, 1967), spawning was reported in March and April. This represents an entirely different cycle, compared to *M. secta* which spawns in August and *M. nasuta* which spawns in May and from September to November. *Ostrea edulis* transplanted in Maine (Loosanoff, 1962) begins its active period in May, avoiding winter buildup entirely. In *Cyprina islandica* (Loosanoff, 1953), gametogenesis occurs principally in the late fall and early winter. There is a slow down in the winter but no dormancy, and it resumes again in spring with increasing temperatures. *Protothaca staminea* (Quayle, 1943) undergoes a short dormancy in December or January after a buildup of follicle cells. However, beginning in January and February active buildup commences despite quite low temperatures. In *Tresus capax* (Bourne and Smith, 1972) another extreme case is found wherein gametogenesis is strong from October through January with an early spring spawn. Here the low winter temperatures of British Columbia's waters have no effect on gametogenesis.

Results (Fig. 3) indicate that *M. nasuta* spawns twice a year in Tomales Bay. In San Francisco Bay, Packard (1918) noted that because of two recruit pulses per year, reproduction of *M. nasuta* was probably semiannual. Having noticed no clear cut year classes in the size—frequency distribution of *M. nasuta,* Addicott (1952) interpreted a year-round spawning of the calm in Elkhorn Slough. Progressing south it appears *M. nasuta* may fulfill a potential of year-round spawning. Coan (1971) noted that *M. nasuta* spawned in the spring or early summer in Oregon. Possibly a threshold temperature for spawning was only reached once (the maximum). This type of behavior has been noted for *Mya arenaria.* Coe and Turner (1938) and others reported one spawning for the clam in New England waters, but Pfitzenmayer (1962) reported two separate spawnings a year further south in Chesapeake Bay. It has been shown that for populations of *Crassostrea virginica* from different geographical areas, physiological variants do occur that respond differently when subjected to conditioning at the same temperature (Loosanoff, 1969).

Rand (1973) has proposed a descriptive model on the temporal aspect of breeding strategies. The environment is represented as the complex vector of its components $e = (e_1, e_2, \ldots)$. Over time he has assigned means and variances to e representing different climates. The function of energy acquisition for both adults and larvae are different according to their physiology, abilities to acquire energy, and environmental grain (Levins, 1968). These functions are in turn dependent on e. Simulation of reproductive strategies of a hypothetical population in different climates indicated the following: northern harsh climates were characterized by a single synchronous spawning per year; temperate climates by two spawnings; and tropical by year round spawning (Rand, 1973).

Synthesis of the literature on reproductive timing of *Mya arenaria* and *Macoma nasuta* on the east and west coast of North America, respectively, indicates general agreement with Rand's (1973) model of changing reproductive strategies along a latitudinal gradient. Both bivalves exhibit a unimodal reproductive pattern in their northern harsher ranges. In their latitudinal midranges both species show a bimodal reproductive pattern. Also, *M. nasuta* apparently spawns year-round in its milder southern range.

That temporal breeding strategies depend integrally on the climate variance may be an important element in sympatric speciation for certain groups. Rand's (1973) model was based on a geographical gradient. However, the same description of breeding strategies should apply to any gradient from physically controlled to biologically accommodated climates (Sanders, 1969).

The intertidal zone provides a similar gradient proceeding from high to low tide zones. In studying the dynamics of high and low intertidal populations of the limpet, *Collisella scabra,* Sutherland (1970) found that high populations (harsh climate) spawned once a year while low populations (mild climate) spawned most of the year. This tendency is also apparent in examining the sympatric species of *Macoma. M. secta* (harsher climate-mid-tide) spawns once a year and *M. nasuta* (mild climate-low tide) spawns twice a year.

This work represents part of a thesis submitted in partial fulfillment for the M.S. degree at the University of the Pacific. I wish to express appreciation to all the members of my committee for use of their personal equipment and libraries, help and interest throughout my study at Pacific Marine Station; Dr. J. Blake, Chairman, and Drs. V. Loosanoff, S. Obrebski, and E. Smith, Director. Two anonymous reviewers helped in the final presentation.

Special thanks are due to Susan for her understanding, and help in the field and at the typewriter.

SUMMARY

1. Spawning probably took two to three weeks for *M. secta* individuals and one to two days for *M. nasuta* individuals. Gonads of inactive *M. secta* are entirely disrupted and hardly recognizable, while gonads of *M. nasuta* remain organized and are filled with vacuolated *follicle* cells. Phagocytosis is pronounced only in spent males of *M. secta.*

2. *M. nasuta* females are characterized by noncellular spherical inclusions in *follicle* cells which are probably nutritive in function. They are numerous and large in size in the inactive stage and decrease in numbers and size as gametogenesis progresses.

3. The *M. secta* population was rather synchronous in its progression through the gametogenic stages and spawned in August. Individuals of the *M. nasuta* population were rather asynchronous in passing through the gonadal cycle; the population spawned in May and from September through November.

4. The null hypotheses of 1:1 sex ratios were accepted for both species. No hermaphrodites were found nor any evidence for sex reversal in either species.

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TENTACLE NUMBER IN CULTURED *HYDRA VIRIDIS*

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The term, *morphogen*, coined by Turing (1952), has recently become popular, and research on morphogens, fashionable. Morphogens are substances involved in morphogenesis (without growth), illustrated by evocators, thought to be active in induction. The recent popularity of the term is derived from its generality, which makes it suitable for formal mathematical treatment and for experimentation. Conceptual simplicity allows investigators simultaneously to pursue the morphogenic consequences of morphogens in computers and their real counterparts in simple experimental situations.

Hydra's linear cylindrical form, marked polarity, with tentacles at one end, a foot at the other, and a budding region near the middle, and its ability to regenerate as well as reproduce vegetatively by budding has attracted the attention of investigators interested in morphogens. The flourishing research on morphogens in *Hydra*, led by Schaller (1973, 1976a,b), has centered on the number of tentacles (tentacle number) produced in pieces of regenerating animals and buds.

Tentacle number is a convenient variable not suffering from the vagueness surrounding criteria for differentiation of hypostome or foot. Independent of diameter or length, a tentacle is identifiable as such. Questions concerning the number of tentacles present on an animal are readily separated from questions concerning their dimensions, and the data on number lend themselves to statistical treatment.

The history of experimental studies on tentacle number has been continuous for more than a decade, and has suggested that tentacle number is a complex variable influenced by many factors. Lesh-Laurie (1974) used tentacle number in her investigations of morphogenic roles of nucleic acids, and Yasugi (1974) demonstrated that the lithium ion promotes the regeneration of entire heads of hydras with reversed polarity, but with an actual reduction in tentacle number. Earlier, Lenicque (1967a, b; Lenicque and Lunblad, 1966) examined the ability of extracts of hydras and inhibitors of neurosecretory substances to alter tentacle numbers during regeneration; and while Lentz and Barrnett (1963) and Lesh and Burnett (1964) did not report counts of tentacle numbers, they clearly indicated an increase in tentacle number in some of the hydras treated with extracts of hydras thought to contain neurosecretory material.

Other factors may also influence tentacle number. Burnett (1961, 1967) has long argued for a morphogenic role of growth in *Hydra* and has experimented with agents that inhibit mitosis, among other activities. Webster (1967) asserts that Colcemid increases tentacle numbers in regenerating animals, but Corff and Burnett (1969) show that colchicine inhibits tentacle regeneration. Shostak and Tammariello (1969) show that the increased tentacle numbers found in Colcemid-treated animals can be due to the retention of buds that would otherwise have detached from parental hydras. Müller and Spindler (1971) attribute the dramatic increase in tentacle number found in animals treated with some of their extracts of hydras to the fusion of potentially separate heads, and Shostak (1977a) shows that fused head-ends can regenerate single heads with abnormally high tentacle numbers.

Tentacle number in normal animals has not been extensively studied. The most frequently cited report on the topic is that of Parke (1900) who concluded that (p. 702) "the number of tentacles possessed by a given Hydra varies in accordance with its age, size and doubtless other factors," and that "the number of tentacles possessed by a bud at the time that it is constricted off, is not usually the same as the number of tentacles that will be possessed by the same individual at the later period." Thus, the tentacle number would seem to be highly variable in individuals and extremely vulnerable to local conditions in the wild.

Hydras can be altered in several ways: via changes in temperature, feeding schedule, and wounding or grafting. Reducing the ambient temperature of a culture tends to increase the size of the average animal (Stiven, 1965; Park and Ortmeyer, 1972; Bisbee, 1973), and the frequency of feeding is well known for its proportional effect on growth (*e.g.*, Shostak, Bisbee, Ashkin and Tammariello, 1968). Wounding and grafting readily alter the size of animals with consequences for regeneration and budding (Shostak, 1972, 1977b).

In the present study, animals cultured under ordinary conditions for our laboratory, first supplied a base line pool of information on normal tentacle numbers and variation in the population. The change in this tentacle number on parental animals and on their own buds was then examined. Feeding schedules, temperature, and wounding and grafting were employed to alter hydras and the effects of these variable on tentacle number in both parents and buds was monitored.

MATERIALS AND METHODS

Specimens of *Hydra viridis* of the large European variety, originally supplied by A. L. Burnett, were cultured in large Pyrex-brand baking dishes at room temperature, 21 to 26° C, fed daily on one to two day old brine shrimp nauplii, *Artemia* sp. (specimens supplied by pet dealer with uncertain species designation), and subjected to a complete change of medium about one hour after feeding (Loomis and Lenhoff, 1956). Experiments and observations were performed on individual animals kept in 55 mm Petri dishes in about 12 ml of culture medium (Shostak, Patel and Burnett, 1965). In experiments involving particular temperatures, animals were kept in incubators and culture medium was brought to the desired temperature before exchanging for old medium. Grafting was performed with the usual methods: human hair was used to skewer the freshly cut pieces together until healing between them was complete (Shostak, 1977b). Statistical methods were based on Snedecor (1956) and Sokal and Rohlf (1969), modified and combined in order to accommodate features of the present experiments. Where no other method is specified, the Student's *t*-test was employed to evaluate differences in paired comparisons.

RESULTS

Tentacle numbers on parental hydras

The first objective was to determine the degree of variation in tentacle number found among hydras selected at random from the mass cultures. The sample of

Table I

Average tentacle number over eight days.

Month Batch	October		November	
	1	2	1	2
	7.9	7.0	7.0	7.0
	8.0	7.0	7.2	6.0
	7.1	7.0	6.0	6.0
	7.0	7.0	7.0	7.0
	7.1	7.0	7.8	6.0
	7.0	7.8	6.0	5.9
	8.0	6.1	6.1	7.0
	8.0	7.0	6.9	6.0
	7.0	6.0	6.0	7.0
	8.0	7.0	7.0	6.0
	6.0	7.0	6.0	7.0
Average for batches	7.38	6.90	6.64	6.44
Average for month	7.14		6.54	
Overall average	6.84			

hydras for study was obtained by taking "standard" animals (having two buds; Shostak, 1968) at random from the cultures and placing them individually in Petri dishes. The animals were maintained for 3 days at which time they were removed, while the buds which had detached that day, were retained. In October and November, 1976, 22 animals in two groups (batches) of 11 were collected. Every animal was fed *Artemia* nauplii in abundance and had its tentacles counted. Different observers scored the animals in the 2 months.

The range of tentacle numbers was 6 to 8 for the sample. The monthly and batch means did not differ significantly, as tested by the F-statistic. In terms of the components of variance, however, months accounted for nearly 36% of the variance, while batches within months accounted for 9%. The remaining variance, that within batches, accounted for more than half the variance. This degree of variation among animals amply justifies statements in the literature that tentacle number in *Hydra* varies under constant conditions and from month to month, and cautions one against attaching importance to small differences.

The next objective was to determine how much variation was due to daily changes in tentacle number on these same animals. Tentacle numbers were scored daily for 8 days, a period thought to be of suitable duration, inasmuch as cellular turnover on tentacles is complete in about 4 days, according to Campbell (1967). The average tentacle numbers for each of the 44 animals observed over the course of 8 days are listed in Table I. Of the 44 animals, 34 showed no change whatsoever, seven showed a change of one, one showed a change of two tentacles, and two animals showed two changes of one tentacle. Most of these changes were transient, however, being reversed on the day following the recorded change. Overall, four

TABLE II

Analysis of variance (two-way).

Source of Variation	Degrees of freedom	Sum of squares	Mean squares	F	Probability
Animals	43	149.2			
months	1	31.4	31.4	5.40	$0.25 > P > 0.10$
batches within months	2	11.6	5.80	2.18	$0.25 > P > 0.10$
animals within batches	40	106.2	2.66	66.50	$0.001 \gg P$
Days	7	0.4	0.06	1.50	$0.25 > P > 0.10$
Error	301	12.4	0.04		

animals lost one tentacle over the 8 days, and one gained a tentacle. The analysis of variance of these data (a two way analysis with the two levels within animals) is summarized in Table II. Variation between months and between batches within months in this table are comparable to the similar statistics calculated for the data accumulated on the first day alone as is the F-statistic. These similarities indicate how little change occurs over the period of eight days. Indeed, the mean squares for days is not significant and represents a very small component of variation. The mean square between animals within the same batch is the only term found to be significant, as one might have anticipated, given the large component of variation due to individuals in the first day's data.

The results emphasize the difficulty one has in attributing significance to small changes in tentacle number between randomly selected animals. The small, for the most part, reversible, and not significant changes in tentacle numbers over the course of a week's study are as reasonably attributed to human error in counting as to actual change. In practice, these experiments, which were done "blind", that is, without prior knowledge of the tentacle number on an animal at the time of counting, may have yielded larger changes in tentacle number than actual, but changes that are, nevertheless, not significant.

During the first week after a bud detaches, it grows and commences budding under the present conditions. The animals nearly double their surface area and begin budding generally by the second day (Shostak, 1968). By the end of the week, most of the animals would be considered "standard", possessing two developing buds. The average animal has produced about four detached buds by this time. Neither growth nor budding, therefore, along with time as such, have altered the tentacle numbers of animals significantly when cultured continuously under these conditions. Tentacle number is a stable characteristic of individual hydras.

Tentacle numbers on buds

The buds produced by the population of 44 parent animals were also scored for tentacle numbers while attached to these parents and on the day of detaching. The range of tentacle numbers on detached buds was 4 to 8, and the mean tentacle number for 403 buds collected was 6.26. Including the 44 parents, which were originally freshly detached buds themselves, 447 buds were collected. Their mean

Table III

Frequencies of tentacle numbers.

Tentacle number	Observed frequencies	Expected frequencies for normal distribution
3	0	0.089
4	3	4.96
5	65	64.77
6	184	197.80
7	174	150.64
8	21	27.54
9	0	1.20
10	0	0

tentacle number was 6.32 with standard deviation 0.79 tentacles. The observed frequencies and those expected from a normally distributed population are given in Table III.

The observed frequencies were tested for skewness and kurtosis using the sample statistics, g_1 and g_2, with their respective standard deviations (Sokal and Rohlf, 1969). Small negative values of both g_1 and g_2 were not significant ($0.4 > P > 0.2$) as shown by the *t*-test with infinite degrees of freedom. The observed frequencies, therefore, are normally distributed, symmetrical and have no significant flattening or peaking compared to a normally distributed population with the same mean and standard deviation.

These conclusions are fairly apparent upon casual inspection of the frequencies in Table III. The observed data appear symmetrical around the values calculated for a normally distributed population. Of course, the "central limit theorem" permits the use of "the normal distribution to make statistical inferences about means of populations in which the items are not at all normally distributed," (Sokal and Rohlf, 1969, p. 130), especially when large sample sizes are employed. The importance of this demonstration of normality is that common parametric statistical tests, such as the F-test for which normal distributions of the sample data are assumed, can now be employed without bias to test inferences about different tentacle numbers between vegetatively produced populations of hydras.

The next objective was to determine if the tentacle number on buds varies as a function of the tentacle number on parents. The correlation coefficient, r, for the 403 buds on the 44 parents studied was found to be significant with a value of −0.14, indicating a loose general relationship between the tentacle number on buds and their parents, which, under the conditions employed, is negative. Actually, two of the three buds with only 4 tentacles were produced by parents having 8 tentacles, the remaining bud with 4 tentacles having been produced by a parent with 6 tentacles. Within the range of tentacle numbers (6–8) found on the present sample of parents, therefore, buds are evidently produced with tentacle numbers varying within a similar range (4–8) and broadly negatively correlated with the parent's tentacle number.

These data also made it possible to determine if tentacle numbers on buds vary with days of the week or the sequence with which buds are produced. Ten buds

TABLE IV

Average tentacle number on developing buds.

Month	Number of animals	Days of bud development on parents detached		
		1	2	3
Oct.	89	2.19	5.92	6.02
Nov.	89	3.54	6.29	6.38
Overall	178	2.86	6.11	6.20

produced on each of 5 weekdays were selected at random from the October and from the November samples. The mean tentacle number for each day did not change significantly as tested by the F-statistic ($0.25 > P > 0.10$) nor did the differences between the means for the two months. To test the possibility that the tentacle number of buds varies as a function of their sequence of formation, 14 parental animals in October and in November, which had complete records for up to the eighth bud, were chosen for the sample. Like days of the week, tentacle number did not change significantly with sequence as tested by the F-statistic. Even the first bud produced by the parents failed to have significantly more or less tentacles than subsequent buds.

The number of tentacles present on developing buds was recorded on each of the parent animals sampled in October and November, providing a record of the progress of each bud. The records of 93 buds in October and 89 in November spanned 3 days (2 days of development on the parent plus the day of detaching) and were used for the samples. (The sample for October was reduced to 89 by randomly eliminating four records in order to have samples of equal size.) Buds that were apparent, but had not yet developed tentacles, were recorded as having a tentacle number of zero. Presumably, when no record of a bud was made 2 days before its detachment, it had not begun developing or had evaded the observer's scrutiny due to small size. This difference would account for buds with records spanning 2 days and those spanning 3 days. No further difference is apparent in the tentacle numbers for these animals. The results are summarized in Table IV.

On inspection, these averages indicate that the greatest period of increase in tentacle number is before the second day of development, after which time tentacle number changes little. The analysis of variance of these data bears out this impression. Significant differences are found between days of bud development. Upon partitioning the variance between Day 1 *vs.* Day 2 and 3, and among Days 2 and 3 (each with one degree of freedom) one discovers that almost all the variance resides between Day 1 and the other days, Days 2 and 3 having only a fraction of the variation between them and a mean square which is not significant. This result pushes back the period during which animals may be said to have stable tentacle numbers to the day before detachment, at least under constant conditions.

Feeding schedule: effect on tentacle number of parents and buds

The frequency of feeding has well known effects on the growth and size of hydras and on the number of buds produced (Shostak *et al.*, 1968). The object of this experiment was to determine if animals fed with different frequencies underwent changes in their tentacle numbers or produced buds with different numbers of tentacles. In addition to the daily feeding schedule, animals were fed on two 3-days a week schedules (intended to approximately halve the caloric input of animals fed daily) and a twice-a-day schedule (intended to double the daily caloric intake). The two 3-days a week schedules were employed to provide animals which were cycling between periods of budding and nonbudding, and animals which were budding at a slow and continuous or diminishing rate. The experiment was carried out for 2 weeks in order to allow an adequate period for changes in tentacle number to develop.

Sets of 12 animals were fed Monday through Wednesday (MTW) and Monday, Wednesday and Friday (MWF) and the tentacles on the parents and on buds counted daily. The results (Table V) for parents and for buds of animals on the two different schedules did not differ significantly. Thus, the average tentacle numbers shown represent the pooled data for parents (column 2) and for buds (column 3). The average tentacle numbers for the parental animals, like the parents fed daily, show little change, a conclusion validated by the analysis of variance. The mean square for days was not significant ($0.75 > P > 0.50$), nor was linear regression ($P > 0.75$). The mean squares for animals within feeding schedules are, however, highly significant, as expected for animals taken at random from stock cultures.

Unlike the parents, the tentacle number on freshly produced buds shows a marked decline after the first week, reaching a minimum by the end of the second week of about one tentacle less than present on buds produced on Day 1. The analysis of variance shows that this change is highly significant ($0.001 > P$) and largely attributable to linear regression, also highly significant ($0.001 > P$). These data were corrected for differences in the sizes of the daily samples (sub-classes). The reduction in the sum of squares due to fitting constants was calculated and the interaction sum of squares obtained by difference from the sum of squares for subclasses (Snedecor, 1956). The results of these calculations had little impact on the analysis, and the interaction term was not significant.

Columns 4 and 5 (Table V) list the number of buds produced by the 12 animals on each feeding schedule on each day of the two week period. The total number of buds produced by animals on each schedule did not differ significantly ($0.10 > P > 0.05$), but the number of buds produced by three of the pairs differed (indicated by asterisks) at the 0.05 confidence level or below when tested by Chi-square. The null hypothesis, that the differences between these paired data are no greater than that expected as a result of random error, is, therefore, rejected in these cases. Feeding 3 days in succession (MTW) seems to have cycled budding so that the detachment of buds peaked on weekends and diminished mid-week. The different rates of budding on animals in the two schedules contrasts with the similarity in the tentacle numbers on the buds, and their decline in tentacle number over time. The tentacle number on freshly produced buds undergoing a reduction in feeding schedule from daily to 3-days a week, therefore, is influenced by caloric intake per week, but not by how the dose of food is supplied.

In another experiment animals were fed twice a day for a comparison with controls fed only once a day. Allowing the animals one week to acclimatize, data on bud

Table V

Average tentacle number on 24 parents and their buds, and number buds produced whole on two feeding schedules.

Day	Tentacle number on parents	Tentacle number on buds	Feeding schedules	
			MTW number buds	MWF number buds
0 Su	6.50			
1 M	6.50	6.48	11	12
2 Tu	6.46	6.50	6	8
3 W	6.46	6.57	7	7
4 Th	6.38	6.35	12	8
5 F	6.42	6.57	11	3*
6 Sa	6.29	6.10	12	9
7 Su	6.33	5.83	13	5*
8 M	6.46	5.75	6	6
9 Tu	6.46	5.80	1	4
10 W	6.46	5.86	4	10
11 Th	6.46	6.41	9	8
12 F	6.46	5.25	7	1*
13 Sa	6.46	5.33	9	3
		Total	108	84

* Difference between paired numbers of buds significant as tested by Chi-square.

production and tentacle number on the seventh and eighth days after commencing the twice-daily feeding schedule were accumulated for analysis. In contrast to the 12 controls which formed 22 buds, the animals fed twice-daily formed 45 buds, twice as many and a highly significant difference. The control population's tentacle number on buds produced was 6.04 and stable around this mean, but the animals fed twice-daily had a mean tentacle number on buds produced of only 5.36, a highly significant difference. Animals fed twice-daily form buds that begin budding themselves sooner, indeed, even while still attached to the parental animals, compared to animals fed once-daily. Thus, feeding animals twice daily to repletion increases size, rate of budding, shortens the time of onset of budding, but decreases tentacle number on buds compared to animals fed only once-daily. The tentacle number on the parental animals of both sample populations did not change significantly over the course of the week, despite the conspicuous change in the size of the animals fed twice-daily.

Effects of changing temperature, wounding and grafting

In addition to excess feeding, the average size of hydras is increased by decreasing temperature (Stiven, 1965; Park and Ortmeyer, 1972; Bisbee, 1973). One of us (D.M.) raised hydras at three temperatures, 16, 21, and 26° C, and determined the tentacle numbers for 12 parents and the buds produced over 8 days at each temperature. In no case did the parent's tentacle number change, but the tentacle numbers of freshly produced buds changed whenever the temperature had changed from the initial condition. The average tentacle number on freshly produced buds on the seventh and eighth days of incubation at the respective temperatures were

TABLE VI

Average tentacle number before and after regeneration.

Number animals	Originally	After regenerations
8	5	6.000*
25	6	5.440*
16	7	5.875*

* Probability that difference resulted from random error less than 0.01.

6.67, 6.33, and 6.04 tentacles per bud. The analysis of variance failed to indicate significant differences among these means, but linear regression was significant and accounted for almost all the variation in tentacle numbers between the means. The regression equation is $Y = 7.65 - 0.062X$, where Y is tentacle number and X is temperature in degrees centigrade. The Q_{10}, or temperature coefficient, is well in the range of expectations for enzymatically mediated biological reactions (Mitchell, 1950), but because fewer tentacles are produced at higher temperatures, suggests inhibition.

Cutting animals also reduces their overall size and ordinarily results in regeneration. When proximal pieces are preserved, tentacles regenerate at their distal wound surfaces. In order to compare the frequencies of tentacle numbers regenerated on proximal pieces of animals cut to different sizes, "standard" animals were cut through their gastric regions (the region between the head and the budding region), either directly below the head (headless animals) or mid-way down the gastric region. The proximal pieces of headless animals are approximately half again as large as the proximal pieces of animals cut mid-gastrically. Regenerated tentacles were counted daily until no further change in tentacle number was recorded (about four days). In a total sample of 82 animals no significant differences appeared in the mean tentacle numbers regenerated by animals cut at either level. The average tentacle number was 6.48 tentacles, despite the differences in size.

This result would seem to differ from that of Schaller (1973) who reports mean differences in tentacle numbers on animals wounded at different levels. However, since she employed nonbudding animals and cut them mid-way down the length of their body columns, she must have wounded them in a considerably lower region than we did here. Furthermore, she does not report that the tentacle numbers achieved were stable. It is well known that head structures in general regenerate at a slower rate at more proximal levels, especially in the peduncle, than at more distal levels of the gastric region (Webster, 1966). Finally, her report is ambiguous as to whether all her animals are starved one or two days prior to wounding, a consideration that could conceivably have significant effects on the frequency of tentacles regenerated inasmuch as feeding schedule effects tentacle number on buds.

In order to determine what relationship might exist between the number of tentacles originally present on an animal and the number regenerated, forty nine "standard" animals, chosen at random, had their tentacle number ascertained and then had their heads amputated. The mean number of tentacles originally present and those present after regeneration was complete are shown in Table VI. The asterisks indicate differences which are significant at the 0.01 level of confidence or below. An interesting feature of this result is that the mean tentacle numbers for all the regenerates do not differ significantly. This suggests that the regeneration of tentacles neither increases nor decreases tentacle number, but tends to regenerate tentacles regressively toward the mean for the population. Animals having tentacle numbers above the mean tend to regenerate fewer tentacles. In this case, the mean tentacle number of the regenerates was 5.57 tentacles. Since the mean tentacle number for the original sample was 6.16, significantly above the mean for regenerates, regeneration does not necessarily restore the mean originally present.

In order to create larger-than-normal animals additional gastric regions were grafted to headless animals thus lengthening the animals approximately a third again as much as they were originally. The distal head, present on the grafted gastric region, was then amputated and the regeneration of tentacles at this wound site monitored. Of 20 animals having an average original tentacle number of 6.45 tentacles, heads regenerated with an average tentacle number of 4.30, a highly significant decrease. In addition, five animals lengthened by grafting failed to regenerate heads at all even after a week. A total of six animals among the 25 regenerated so-called secondary heads (Shostak, 1972) at the border between the grafted pieces. The mean tentacle number for these secondary heads was 4.17 tentacles, not significantly different from the mean tentacle number regenerating on heads at the distal ends of the animals. Animals elongated by grafting, therefore, regenerate significantly fewer tentacles than normal at both distal wound sites and at graft borders.

Headless animals may also be grafted together in a "head-to-head" fashion, thus making an animal about twice the normal length, but with feet at both ends. Such animals regenerate heads at the border between the graft pieces, and in about half of the animals form a single integrated head rather than two heads (for a similar case see Shostak, 1977a). For 18 such grafts regenerating single heads, a mean tentacle number of 8.50 was found, a highly significant increase compared to the mean of 6.11 for the original heads, prior to amputation, but, certainly nowhere near a doubling of the original tentacle number. Among these, animals with 9 to 11 tentacles were selected (a sample of 7) and had their new heads amputated. Beginning with a mean tentacle number of 9.57, these animals regenerated tentacles with a mean tentacle number of 8.29, a significant diminution. Here again, tentacle numbers of regenerates tend to regress toward a normal value, but evidently, the diminution of tentacle numbers only proceeds at the rate of one to two tentacles per regeneration.

DISCUSSION

Hydra has long figured in the pursuit of morphogenically significant substances, but has recently received wide spread attention due to the success of studies apparently demonstrating the morphogenic activity of neurosecretory substance. Schaller (1973; 1976a, b) has reported fractional increases in the mean tentacle numbers of samples of regenerating animals exposed to what are reported to be moderately purified neurosecretory products, compared to samples of other untreated regenerating animals. In order to evaluate such reports, one would like to know how tentacle number is distributed in the normal population and how its mean and variation are determined.

The present study agrees with two earlier studies (Parke, 1900; Liu and Chang, 1946) that tentacle number varies widely in populations of *Hydra*. Parke, who studied *H. fusca* and *H. viridis* from the wild, however, seems to have had difficulty culturing these animals and acknowledges the occurrence of greatly distorted animals whose regulation of form is discussed at length. Liu and Chang studied *H. vulgaris* and, as in the present study of *H. viridis*, found tentacle number normally distributed in their population. Since the introduction of adequate methods for mass-culturing *Hydra* in the laboratory by Loomis and Lenhoff (1956) the appearance of abnormal animals is rare, especially in rapidly budding colonies not heavily infested with endemic parasites. The variation of tentacle number found in the present study, therefore, is not attributed to poor conditions or variations found among wild populations. Even under vigorously controlled laboratory conditions, the tentacle number of *H. viridis* is normally distributed.

The present report also confirms Liu and Chang's (1946) conclusion that tentacle number is fixed prior to bud detachment, indeed, as much as a day prior to detachment. Following this time, an animal's tentacle number may be considered a stable characteristic, barring amputation or the extreme conditions known as depression, which presumably affected Parke's animals. Animals in the present study resisted changes in tentacle number for periods of observation as long as two weeks during which feeding was reduced or temperature was altered. Tentacle number seems to be fixed with a degree of determination which suggests structural rigidity. Variation in tentacle number found in the populations produced under adequate circumstances cannot be attributed to changes in individuals' tentacle numbers, but must be due to variation resulting from the production of buds with different tentacle numbers.

The freshly detached buds collected in the present study had tentacle numbers which were normally distributed. Evidently, the development of tentacles on buds is subject to controls that concentrate animals at the mean tentacle number and provide fewer animals with other tentacle numbers. One might expect that the control of the mean tentacle number is genetic and inherited and that variation is due to random error around a mean. The present results on the effect of size, temperature, feeding schedule, and wounding and grafting indicate that setting the mean tentacle number is more complex.

A variety of conditions were found to alter the mean tentacle number of freshly produced buds. We were especially interested in conditions which were known to alter the size of the budding animal, since Parke (1900) had reported that tentacle number varied with size and changed as a function of size. Otto, Dunne, Wirth, and Campbell (1976) and Otto and Campbell (1977) showed that the size of hydras is correlated with the size of buds, albeit they failed to report on tentacle numbers. The present study, likewise, shows that larger animals, cultured at 16° C, produced buds with more tentacles than smaller animals, cultured at 26° C. Such results are clearly suggestive of so-called mass phenomena in which the density, mass, or volume of cells present at a time has its own morphogenic consequences. The negative relationship between temperature and tentacle number suggests that metabolic rate is inversely proportional to tentacle development, or that products of metabolism are inhibitory to tentacle formation. The rate at which animals grow, determined by the frequency at which they are fed, controls the rate of budding and may have indirect consequences for tentacle development. Animals fed more frequently than once a day bud so rapidly that buds begin budding before actually detaching from their parents. Growth that is overwhelmingly directed to budding may divert cells into buds that are otherwise destined to take part in tentacle formation. When animals are fed less frequently than once a day, fewer cells may be available for recruitment into tentacles. The genetic constitution of parental animals is difficult to contemplate in the absence of specific information, but since buds are produced vegetatively one would expect that the buds and parents would have the same genetic complexion. Our finding that the tentacle number on buds is negatively correlated with the tentacle number of parents in the sample of animals tested suggests just how complex the problem of genetic control of tentacle number may be. Finally, as was first pointed out by Liu and Chang (1946), a biological parameter which is normally distributed, with a mean and a standard deviation, is inevitably genetically determined although the mechanism for fixing tentacle number in any animal may be subject to the effects of polygenes and modifier genes as well as to environmental conditions. The setting of a population's mean tentacle number, therefore, would seem to represent the culmination of the interactions of a variety of factors.

Thresholds would presumably be a part of any endogenous control system generating buds with different numbers of tentacles. Such a control system has already been hypothesized to operate in determining the production of a hydra's foot (MacWilliams, Kafatos, and Bossert, 1970). There, an inhibitor of foot formation is thought to be released by the foot with continuously variable intensity controlling foot regeneration and normal maintenance. Given this perspective, one can imagine an inhibitor of tentacle number whose quantitative production is sensitive to physiological and environmental cues, but which operates a mechanism inhibitable at given threshold levels of concentration. One level might determine that six tentacles are produced, another level that five tentacles are produced and so on. The neurosecretory substances investigated by Schaller (1976a, b) are not thought to play inhibitory roles, however. Such a role may be played by another substance in her scheme. Rather, neurosecretory substances are thought to promote or determine tentacle development. Alternatively, physiological and environmental cues might alter thresholds rather than levels of inhibitors or determinants, or both thresholds and levels of substances responsible for controlling tentacle number. This possibility would seem to have sufficient modality to encompass the present results.

A complex system for determining tentacle number would seem well suited for hydra's way of life, which subjects it to wide and repeated variations in environmental conditions. Presumably, tentacle number is a crucial adaptation for hydras in the wild and is, therefore, subject to natural selection and hereditary control. Once fixed, a bud's tentacle number remains constant except under conditions initiating regeneration. An optimal tentacle number in one environment may not be optimal in another, however, and a system for changing the mean tentacle number would also be adaptive. We have seen that the animals generate buds with different tentacle numbers which, by chance might have selective advantages in terms of leaving progeny in the different environments they encounter. Natural selection might then shape the population preferentially according to local conditions, allowing animals with the optimal tentacle number for these conditions to survive while eliminating animals with nonoptimal numbers. Parke (1900), not only observed that the tentacle numbers of *H. fusca* differed from those of *H. viridis,* but different populations of members of the same species taken from different locales also had different mean tentacle numbers. But, *Hydra* is not totally dependent on random variation for generating animals with different tentacle numbers. The mean tentacle number on buds produced under different conditions is capable of shifting in predictable ways. Replacement populations produced by budding may have mean tentacle numbers more nearly at the optimal level for particular local conditions, and contain fewer individuals with nonoptimal tentacle numbers than would be produced merely by a random system for generating variation.

Regeneration of tentacles also seems adaptive for altering the mean tentacle number of a population. The number of tentacles regenerating on distal pieces tends to gravitate toward a mean, characteristic of the population, not necessarily resembling that of the original population, and not conspicuously sensitive to size. The number of tentacles regenerating on wounded gastric regions can actually exceed that number originally present when that number was above the mean. Even animals with abnormally high numbers of tentacles (produced by the production of a single head from two regenerating surfaces brought together by grafting) have fewer tentacles than originally present on the two heads separately, and regenerate tentacles with more nearly normal tentacle numbers than they had prior to amputation. *Hydra,* thus, seems to "know" how many tentacles to produce in a given environment and under particular physiological conditions, and regenerates tentacles in increasingly close approximations to that number as opportunities made available by budding and amputation permit.

Regeneration is conspicuously similar to budding as a nexus for altering tentacle number. Furthermore, like the hydras enlarged by feeding twice a day which produce buds with fewer tentacles than animals fed once a day, hydras enlarged by grafting regenerate fewer tentacles than pieces of normal animals. But, unlike budding, the role of regeneration in the wild is unknown. Except for adverse conditions causing depression and subsequent regulation, regeneration as such may be infrequent in wild populations. Budding, rather than regeneration would seem to be the general route for regulating tentacle number in wild populations.

The present study should have some impact on those studying morphogens, whether simple or interacting, inhibitors or stimulators, especially those employing regenerating hydras as their test system. If hydras are "imperfect test tubes" (Schaller, 1976a, p. 1), it is because *Hydra* has evolved mechanisms for moderating the effects of environmental contingencies. *Hydra* may meet challenges, both in the wild and in the laboratory, in ways contrary to expectations. Relying on its own wisdom, *Hydra* has evolved systems for rigidly maintaining the tentacle number of individuals despite moderate changes in environmental conditions, for generating buds with a normal distribution of tentacle numbers, and for regenerating heads with tentacle numbers that may be different from those originally present. Among the things a hydra "knows" is how to produce buds with different mean numbers of tentacles and to regenerate tentacles in numbers that increasingly approximate a mean dictated in part by particular conditions. One might be rewarded by efforts to understand a hydra's wisdom in stabilizing tentacle numbers on individuals while providing a mechanism for generating variety and shifting means.

The present report makes clear, however, that relying on small fractional differences in tentacle numbers to draw large inferences about the operation of morphogenically significant substances in *Hydra* is not prudent.

SUMMARY

1. The distribution of tentacle numbers on *Hydra viridis,* cultured in the laboratory were studied in order to gather some appreciation of the normal range of tentacle number, and how that number is influenced by vegetative reproduction, and by contingencies which alter the size of a hydra.

2. Large variation among individuals selected at random was found to be the predominant statistical factor in the analysis of variance of tentacle number and cautions against drawing inferences from small differences in sample mean tentacle numbers. Tentacle number is normally distributed and stable for each individual from the day before detaching as a bud for periods as long as 2 weeks despite changes in feeding schedule and temperature.

3. The mean tentacle number on samples of buds produced over time is subject to change. Tentacle number on buds can be negatively correlated with tentacle number on parents and decreases both when the normal daily feeding schedule is changed to a 3-days a week schedule (whether in succession or with intervals between feedings), or increased to twice-daily feeding. Buds produced at higher temperatures also have lower tentacle numbers. Conditions which might raise the metabolic rate of the parent hydras, therefore, can reduce tentacle numbers on buds.

4. Hydras wounded through the gastric region regenerate the same number of tentacles despite differences in size. Regeneration yields tentacle numbers tending to gravitate toward a mean for the population independent of the original tentacle number. Both increases and decreases are recorded. Animals enlarged by grafting, however, tend to regenerate fewer tentacles than originally present even when double-heads regenerate as one. Regeneration, like budding, can alter the population's mean tentacle number according to prevailing conditions.

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CHROMOSOME KARYOTYPES OF THREE BIVALVES: THE OYSTERS, *ISOGNOMON ALATUS* AND *PINCTADA IMBRICATA,* AND THE BAY SCALLOP, *ARGOPECTEN IRRADIANS IRRADIANS*[1]

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Since Menzel and Menzel (1965) reported on the chromosomes of two species of clams, several workers have observed the chromosomes of bivalves (pelecypod molluscs) using squash or air-drying techniques (Ahmed and Sparks, 1967, 1970; Longwell, Stiles and Smith, 1967; Menzel, 1968; Patterson, 1970; Ieyama and Inaba, 1974; Ieyama, 1977). However, there are still only a few species of bivalves for which chromosome numbers have been established, as compared with the greater numbers of gastropod molluscs with known chromosome karyotypes.

This paper presents the chromosome number and gross morphology of three species of Bivalvia not previously reported: *Isognomon alatus,* the flat tree oyster; *Pinctada imbricata,* the Atlantic pearl oyster; and *Argopecten irradians irradians,* the commercial bay scallop.

MATERIALS AND METHODS

Specimens of *Isognomon alatus* were collected in June, 1976, from Biscayne Bay, Miami, Florida. Specimens of *Pinctada imbricata* were collected in February, 1977, near Pompano Beach, Florida. Cultured specimens of *Argopecten irradians irradians* were obtained from a commercial hatchery on Long Island, New York.

Eggs and sperm were obtained by stripping the gonads of *I. alatus* (10 animals). Spawning was induced thermally in *P. imbricata* (14 animals) and in *A. i. irradians* (25 animals). *A. i. irradians* is hermaphroditic and usually spawns both eggs and sperm almost simultaneously. When unfertilized eggs were needed, these animals were induced to spawn in sea water containing 0.02% EDTA (ethylene-diaminetetraacetic acid) which, by chelating calcium out of the sea water, must prevent the acrosome reaction of the sperm essential for fertilization. Self-fertilization was effectively prevented by the EDTA. EDTA-exposed eggs were washed with fresh sea water just after spawning, and no chromosome damage was observed in them. Following fertilization, eggs and embryos were fixed at intervals in Carnoy fixative (3:1). Chromosome preparations were made by squashing the eggs or embryos in 1 to 2% aceto-orcein.

To obtain colchicine metaphase in some cleaving eggs, fertilized eggs were held in sea water containing 0.02% colchicine for 15 to 30 minutes. *P. imbricata* eggs were not treated with colchicine.

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[1] Contribution No. 245 from the National Pearl Research Laboratory, Kashikojima, Mie, Japan.

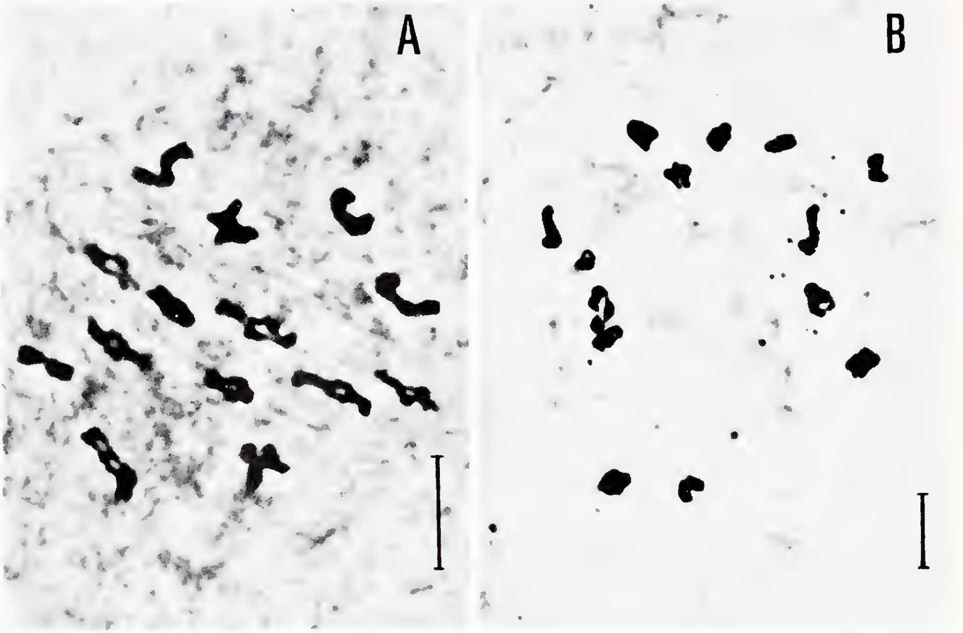

FIGURE 1. Meiotic chromosomes of *Isognomon alatus:* A, fourteen metaphase I chromosomes; B, fourteen metaphase II chromosomes. Scale bars represent 5 μ.

Chromosomes were examined with a 100X Zeiss phase-contrast objective, and suitable cells were photographed. Drawings were made to supplement the photographs.

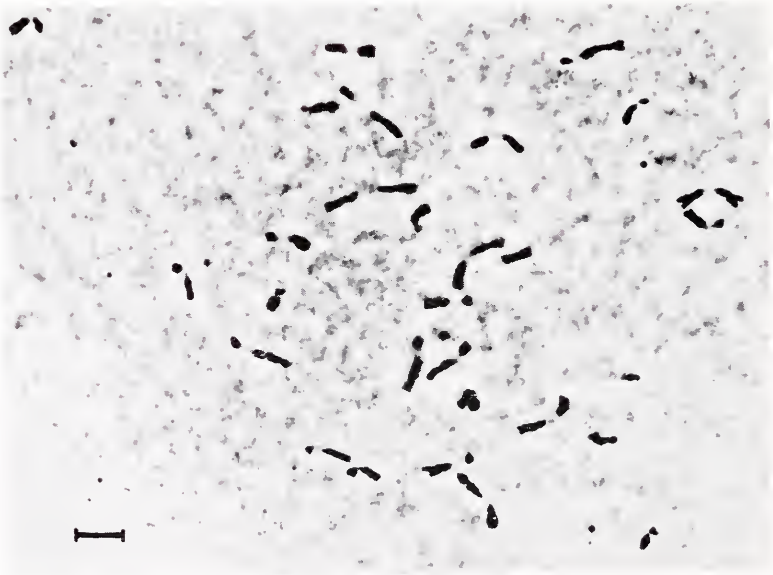

FIGURE 2. Twenty-eight colchicine-metaphase chromosomes of first cleavage in *Isognomon alatus.* Scale bar represents 5 μ.

OBSERVATIONS AND RESULTS

Flat tree oyster, I. alatus

Stripped and unfertilized eggs were oocytes at meiotic prophase I about diakinesis. After insemination, eggs proceeded to complete meiosis. Clear counts of 14 pairs of chromosomes were observed in 10 eggs at metaphase I (Fig. 1A) and 14 chromosomes in 14 eggs at metaphase II (Fig. 1B). The chromosome pairs are all homomorphic and isopyknotic with no evidence of specialized sex chromosomes. Chiasmata are present, indicating the occurrence of crossing-over of the genes. The diploid number of 28 was observed in well-spread cleavage metaphases or anaphases from both 30 embryos treated with colchicine and from 20 untreated embryos. Figure 2 shows a well-spread metaphase plate of a colchicine-treated embryo.

Five sets of 28 chromosomes in colchicine metaphase were tentatively arranged into pairs according to decreasing size. Total measured length of these chromosomes, arm ratio and general morphological appearance, as measured and observed in the photographic prints, were the criteria used for pairing. Using the mean and standard deviation of the 5 tentative arrangements in Figure 3, mean length extended from 6.0 ± 0.78 to 2.2 ± 0.03 μ or from 5.6 ± 0.25 to $2.1 \pm 0.07\%$ of the total length of the diploid complement. Arm ratios measured from 1.2 ± 0.01 to 2.6 ± 0.24. Five chromosome pairs may be tentatively classified as metacentrics and the others as submetacentrics (Levan, Fredga and Sandberg, 1964). Both metacentrics and submetacentrics are included among the longer and the shorter chromosomes. Apparent variation in chromosome size is probably due to nonsynchronous coiling of the chromosomes, both natural and colchicine-induced (see Fig. 3).

Atlantic pearl oyster, P. imbricata

Spawned and unfertilized eggs were at diakinesis of metaphase I. Fourteen homomorphic and isopyknotic chromosomes were counted in about 200 squashes of eggs at metaphase I (Fig. 4A). Chromosomes complete meiosis after insemination, and 14 chromosomes were observed in 24 eggs at metaphase II (Fig. 4B).

Twenty-eight chromosomes were counted in well-spread metaphase or anaphase plates in 8 first cleavage embryos untreated with colchicine. Figure 5 shows metaphase plates of first cleavages.

The chromosomes in drawings of two mitotic metaphase plates were tentatively arranged into 14 pairs in the same manner as done for *I. alatus* (Fig. 6). Mean length ranged from 7.8 ± 0.99 to 1.7 ± 0.44 μ (mean and standard deviation of two tentative arrangements in Fig. 6). Three or four pairs can be classified tentatively as subtelocentrics and the others as submeta- or metacentrics. These are, however, only preliminary observations, and there are very sizeable morphological variations of the chromosomes in the arrangement.

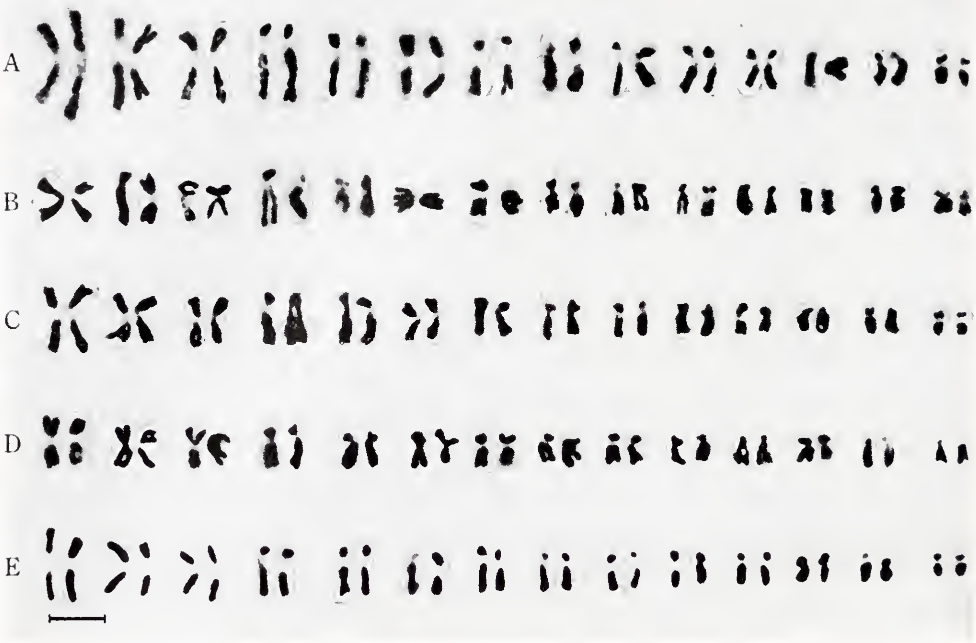

FIGURE 3. Five tentative arrangements of colchicine-metaphase chromosomes of early cleavages in *Isognomon alatus*. Scale bar represents 5 μ.

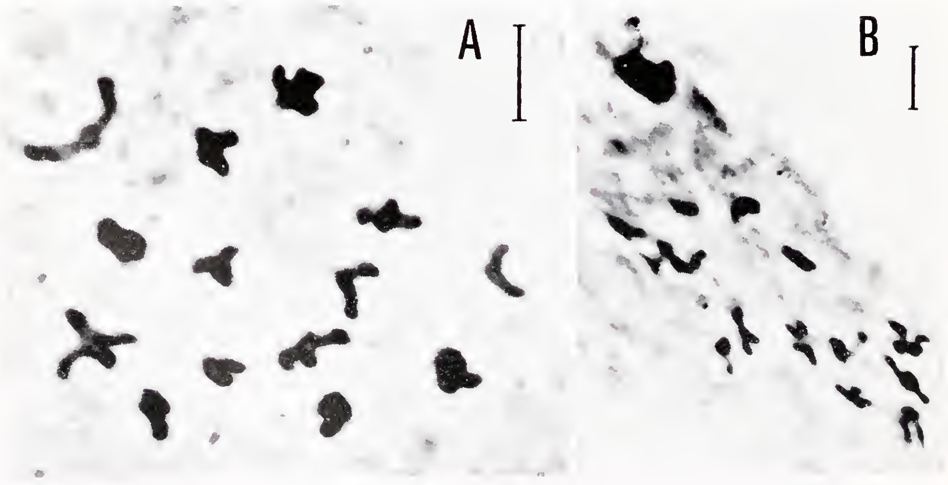

FIGURE 4. Meiotic chromosomes of *Pinctada imbricata:* A, fourteen metaphase I chromosomes; B, fourteen metaphase II chromosomes and first polar body. Scale bars represent 5 μ.

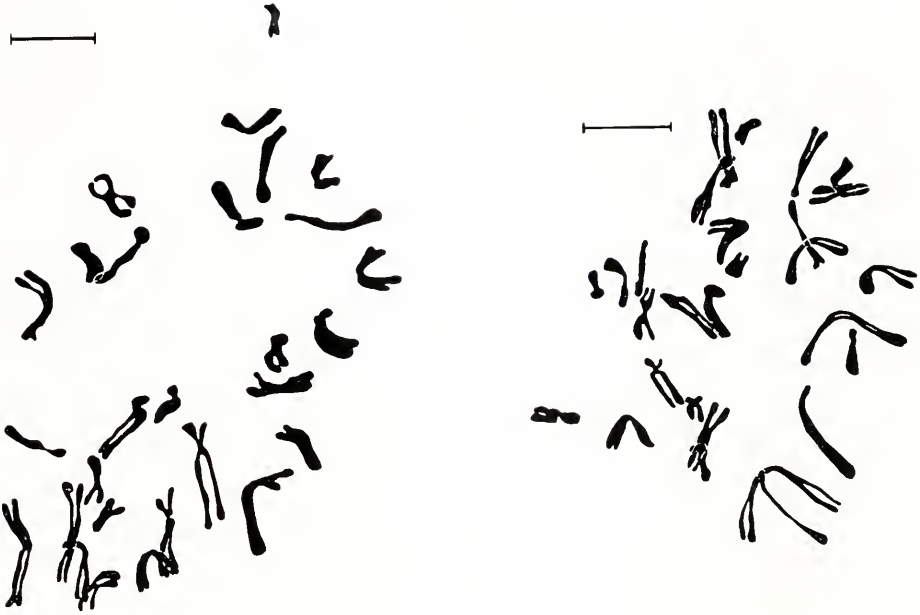

FIGURE 5. Two drawings of mitotic metaphase plates with twenty-eight chromosomes of first cleavages in *Pinctada imbricata*. Scale bars represent 5 μ.

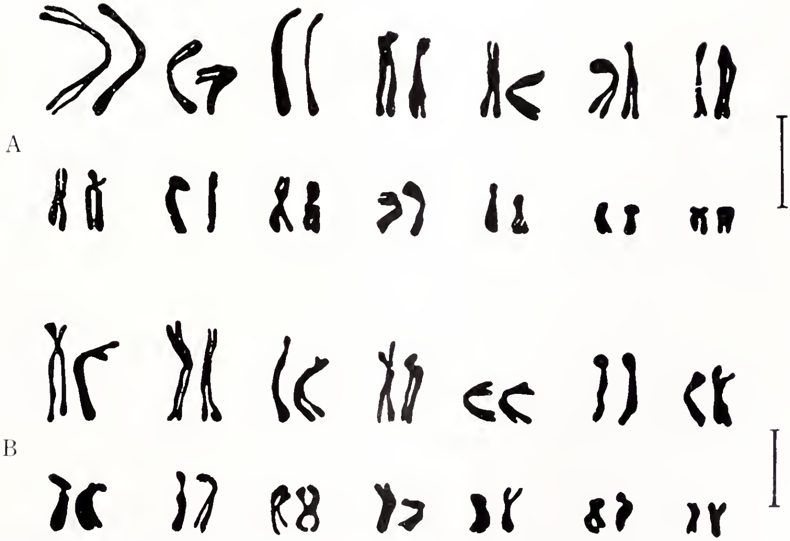

FIGURE 6. Two tentative arrangements of mitotic metaphase chromosomes of *Pinctada imbricata* from the drawings of Figure 5. Scale bars represent 5 μ.

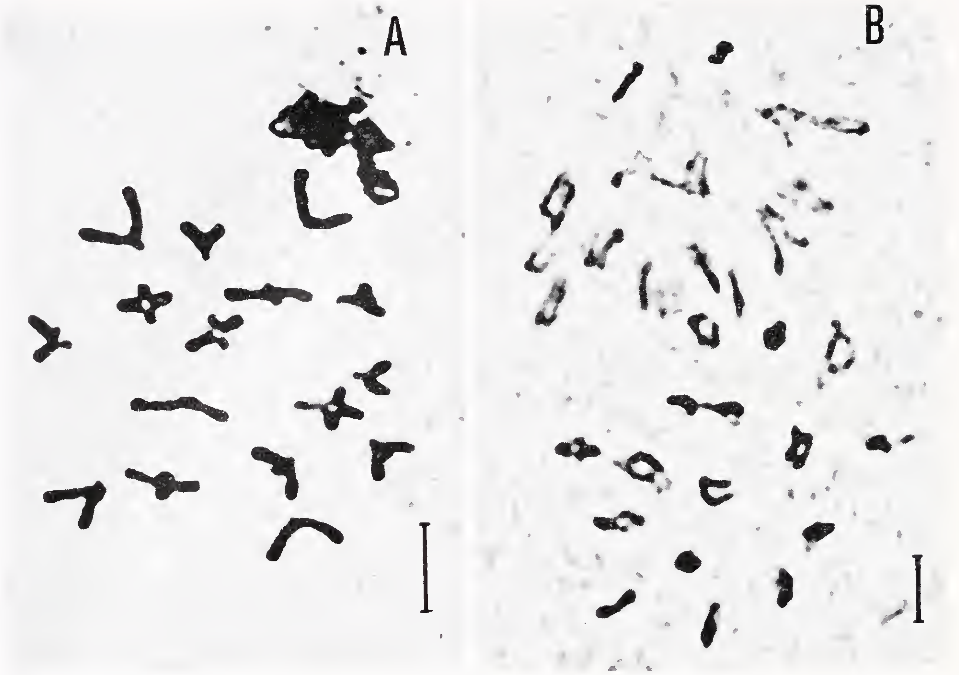

FIGURE 7. Meiotic chromosomes of *Argopecten irradians irradians:* A, sixteen metaphase I chromosomes; B, sixteen pairs of anaphase I chromosomes. Scale bars represent 5 μ.

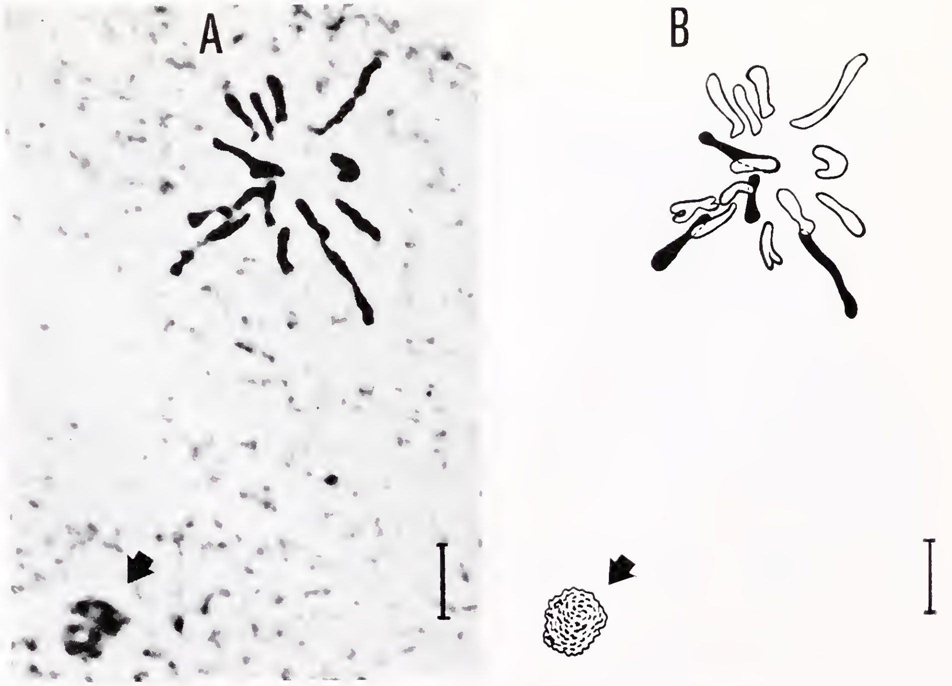

FIGURE 8. Meiotic chromosomes of *Argopecten irradians irradians:* A, sixteen metaphase II chromosomes and sperm nucleus (at arrow); B, drawing of the chromosomes shown in A.

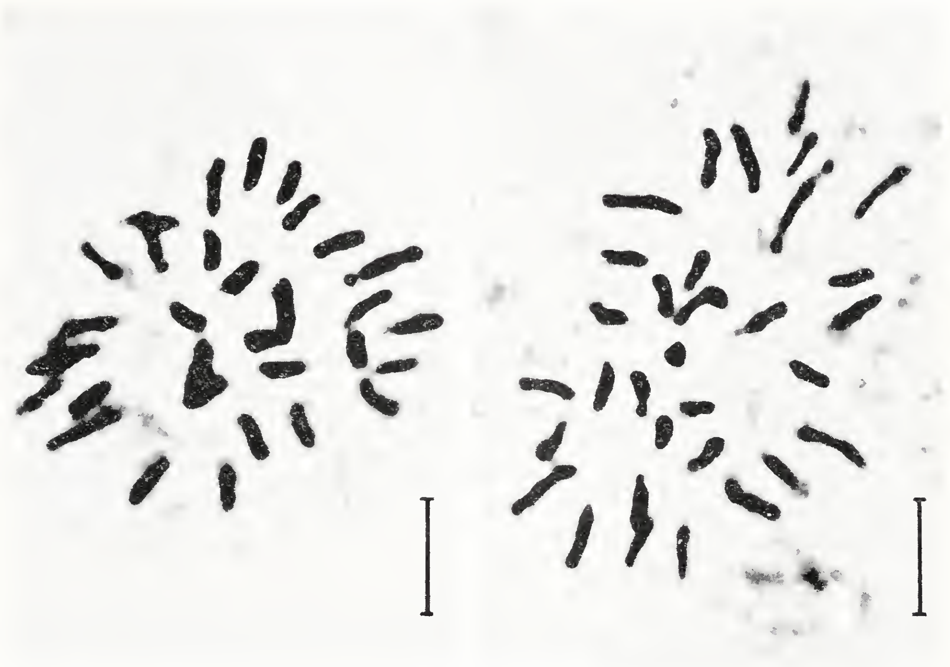

FIGURE 9. Thirty-two colchicine-metaphase chromosomes of first cleavages in *Argopecten irradians irradians.* Scale bar represents 5 μ.

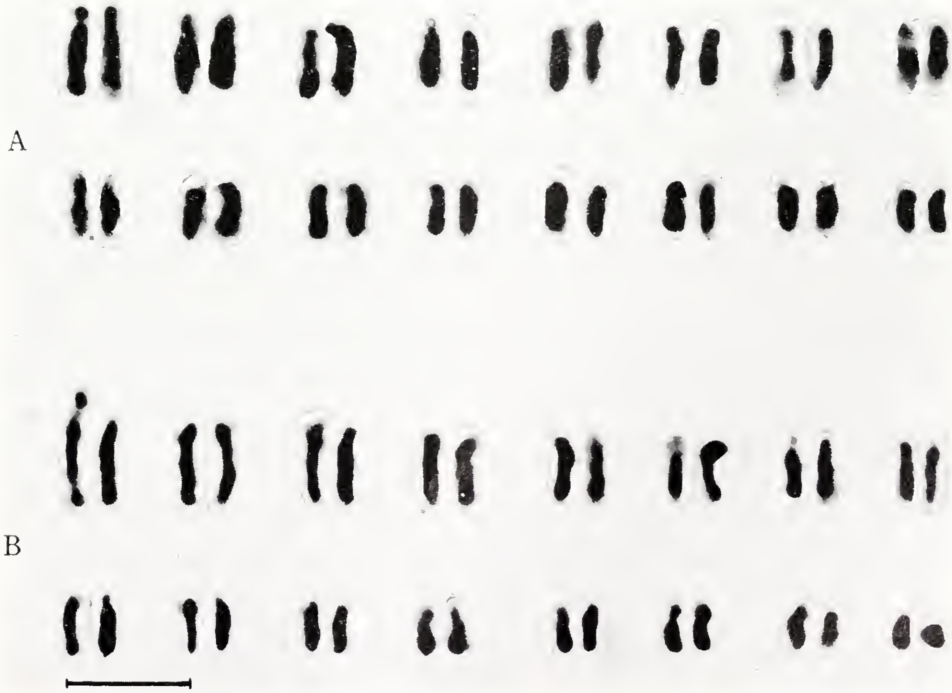

FIGURE 10. Two tentative arrangements of colchicine-metaphase chromosomes of first cleavages in *Argopecten irradians irradians.* Scale bar represents 5 μ.

Bay scallop, A. irradians irradians

Unfertilized eggs were at diakinesis or metaphase I. Sixteen homomorphic, isopyknotic chromosome pairs were observed in over 50 squashes of meiosis I at metaphase (Fig. 7A). (Figure 7B shows these chromosomes at anaphase I.) After insemination 16 chromosomes were counted in 6 eggs at metaphase II (Fig. 8).

Thirty-two chromosomes were counted in well-spread metaphase plates in 30 embryos treated with colchicine and in 20 untreated ones. Figure 9 shows colchicine metaphase plates of first cleavages.

The chromosomes in Figure 9 were tentatively arranged into 16 pairs (Fig. 10). It was difficult to recognize the centromere in some chromosomes, and there were large morphological variations because of the technique used. Even so, the chromosome complements seemed to consist of meta-, submeta-, subtelo- and telocentrics. Mean length ranged from 5.0 ± 1.67 to 1.8 ± 0.23 μ or from 5.2 ± 0.26 to 1.9 ± 0.30% of the total length of the diploid complement (mean and standard deviation of four tentative arrangements). The construction in one of the longest chromosomes might have been a technical artifact.

Discussion

There has been no report to date on the chromosome number of any species in the family Isognomonidae of the order Pterioida. The chromosome number ($2n = 28$) of *I. alatus* given above coincides with those of *Pinctada fucata* (Wada, 1976) and *P. imbricata* also given above. All of these species are in the superfamily Pteriacea, and are the only ones examined in this superfamily (Table I). Constancy of chromosome number here seems to be one additional example of the tendency for chromosome number to be stable within the family or superfamily of Pelecypoda (Menzel, 1968; Patterson, 1970; Ieyama and Inaba, 1974).

In the Japanese pearl oyster, *P. fucata,* about four of 14 pairs in the tentative arrangement of early cleavage metaphase chromosomes were identified as subtelocentrics and others as meta- or submetacentrics (Wada, 1976). In the study of the Atlantic pearl oyster reported here, three or four subtelocentrics were likewise observed, and the others were similarly meta- or submetacentrics. No one has previously compared the chromosomes of the Atlantic and the Japanese pearl oysters. In his taxonomic revision of the genus Pinctada, Ranson (1961) classified the Atlantic pearl oyster, which also has distribution in the Pacific Ocean, as well as the Indian Ocean, Persian Sea and Mediterranean Sea, as *Pinctada radiata* (Leach). He classified the Japanese pearl oyster, *P. martensii* (Dunker), separately from *P. radiata.* Recently, Kuroda, Habe and Oyama (1971) reported the Japanese oyster to be a subspecies, *P. fucata martensii* (Dunker), of the tropical Pacific oyster, *P. fucata* (Gould), which was reported to be synonymous with *P. radiata* by Hynd (1955). Hayes (1972) considered the Atlantic species to be a synonym of *P. imbricata* Röding. The similarity of chromosome number in the Japanese and Atlantic pearl oysters may have bearing on their taxonomy. It would be interesting to observe if chromosome behavior at meiosis in a hybrid of these two oysters appeared to be normal or abnormal. The latter would indicate chromosome dissimilarities in spite of karyotypic similarity.

TABLE I

Chromosome numbers of three families of pelecypod molluscs.

Species name	2n	n	Reference
Isognomonidae			
Isognomon alatus	28	14	Present study
Pteriidae			
Pinctada fucata	28	14	Wada, 1976
P. imbricata	28	14	Present study
Pectinidae			
Pecten maximus	38	19	Beaumont & Gruffydd, 1974
Chlamys varia	38	19	Beaumont & Gruffydd, 1974
Ch. distorta	38	19	Beaumont & Gruffydd, 1974
Ch. islandica	38	19	Beaumont & Gruffydd, 1974
Ch. opercularis	26	13	Beaumont & Gruffydd, 1974
Argopecten irradians irradians	32	16	Present study
Placopecten magellanicus	38	19	Beaumont & Gruffydd, 1974

In the family Pectinidae, the following chromosome numbers of six species were reported (Beaumont and Gruffydd, 1974): 2n = 38 for *Pecten maximus, Placopecten magellanicus, Chlamys varia, Ch. distorta* and *Ch. islandica* and 2n = 26 for *Chlamys opercularis.* The number (2n = 32) for *A. i. irradians* examined in the present study is different. Beaumont and Gruffydd (1974) implied that the mode of diploid number for the family Pectinidae was 2n = 38. They considered the possibility that the number of *Ch. opercularis* (2n = 26) was probably the result of "Robertsonian" centric fusion and that this species derived from an ancestral type with more than 26 (2n) chromosomes. At any rate, there seem to be some variations in chromosome number among species and genera within the family Pectinidae. This is in contrast with the tendency of chromosome numbers within other families of Bivalvia to be the same, as noted above. However, to confirm the seeming chromosome variation, further studies are needed on other species of this large family Pectinidae, which includes about 350 species (Hertlein, 1969).

A. i. irradians is hermaphroditic and the absence of defined sex chromosomes is expected. Self-fertilized and cross-fertilized embryos alike appear to develop with equal success in the laboratory, although self-fertilization is uncommon in nature (Castagna and Duggan, 1971; Castagna, 1975). No difference in incidence of chromosome irregularities in self- and cross-fertilized embryos was apparent on cursory examinations. More study would be needed, though, to confirm this impression.

I wish to express appreciation to Dr. Arlene Longwell of the Milford Laboratory, National Marine Fisheries Service, for her comments and critical evaluation of the manuscript; and to Dr. James E. Hanks, Director of the Milford Laboratory, for his permission to conduct this study at Milford. I am also grateful to Messrs. George Miller (Miami Laboratory, NMFS), Edwin W. Rhodes (Milford Laboratory) and Andre LaBonte for their collection and transportation of the flat tree oyster and the Atlantic pearl oyster. Thanks are also expressed to Miss Rita S. Riccio for her critical reading and typing of this manuscript.

SUMMARY

Chromosome number and morphology were observed in the squashed eggs and embryos of three bivalve species, *Isognomon alatus*, *Pinctada imbricata* and *Argopecten irradians irradians*. *I. alatus* has 14 pairs of chromosomes, all of which are either meta- or submetacentrics. Three or four of 14 chromosomal pairs of *P. imbricata* are subtelocentrics and the rest are submeta- or metacentrics. The chromosome complement of *P. imbricata* seems to be similar to that of *P. fucata*. Chromosome numbers of *A. i. irradians* are 16 (n) and 32 (2n) and are different from those of six Pectinidae species previously reported. The 16 pairs of chromosomes of *A. i. irradians* appear to consist of meta-, submeta-, subtelo-, and telocentrics.

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UNDERWATER ORIENTATION IN THE SAND FIDDLER CRAB, *UCA PUGILATOR*

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The sand fiddler crab (*Uca pugilator*) inhabits the intertidal and supratidal zones of sheltered sandy coastlines from Cape Cod to Texas (Crane, 1943). As with other ocypodids, its semi-terrestrial designation stems from its physiological dependence upon periodic submersion and from its behavioral activity on aerially-exposed substrates. Burrows dug in the sand serve as shelter during predatory attacks and high tides, as immediate sources of water, and as focal points of social activity. Occasionally, a crab is forced to seek refuge in the water when access to the burrows is blocked by pursuing predators such as plovers, willets, raccoons, clapper rails, etc. (Herrnkind, 1972; Teal, 1958; and personal observation). It then becomes vulnerable to attack by such aquatic predators as fish and the portunid crabs, *Callinectes sapidus* and *Carcinus maenas*. Hence, it would be of selective advantage for *Uca pugilator* to possess some means of detecting the direction of the shore and a suitable burrow while submerged. Indeed, field releases of free-ranging *U. pugilator* in shallow offshore waters result in marked orientational movements toward shore (L. M. Lutton and D. Y. Young, unpublished data; W. F. Hernkind, unpublished data).

Terrestrial orientation in the genus *Uca* has been investigated only in recent years (Altevogt, 1965; Altevogt and von Hagen, 1964; Herrnkind, 1966, 1967, 1968, 1972). A thorough review of orientation in shore-inhabiting arthropods, with specific reference to *Uca pugilator,* is given by Herrnkind (1972). On aerially exposed beaches *U. pugilator* utilizes a time-compensated sun compass, supplemented by telotactic responses to landmarks for directed movements of distances greater than one meter ("far orientation"). Kinesthesia of substrate features is not known to influence orientation over these distances. Instead, kinesthetic cues are said to be limited to above water movements of less than one meter ("near orientation") (Herrnkind, 1972).

Orientation cues available to the crab underwater potentially differ from those on land due to differences in the physical characteristics (*e.g.,* refractive index, specific gravity, transparency, etc.) between sea water and air. Visual cues, such as those emanating from celestial and landmark sources, may be available to submerged crabs as well as to those situated on the exposed portions of the beach. Celestial cues involve the sun's position and/or polarized light. Landmarks could be detected as differential light intensities created by beach grasses, trees, and mangroves set against a sky or sand background. In both cases, the apparent position of the cues is refractively modified by the air-water interface. These cues are also subject to distortion by variable water surface conditions. Sea water, having a specific gravity and light absorptive index greater than those of air, may also allow submerged crabs to use some gradient cues for shoreward orientation. Any vertical movement in water will result in greater changes in ambient pressure and light intensity than those ensuing from an equal movement in air. Theoretically, movements along a decreasing pressure gradient would eventually allow a submerged crab to reach land, even in the absence of celestial and landmark cues. Appropriate movements along gradients of light intensity and/or light wavelength will similarly result in the crab's leaving the water. Likewise, upward movements along substrate slopes will generally lead out of the water.

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Many animal orientation systems entail redundant schemes of cues in which certain stimuli, or combinations thereof, dominate others in a hierarchial or synergistic fashion (Adler and Taylor, 1973; Bellrose, 1965; Ferguson, 1965; Keeton, 1971; Papi and Pardi, 1953; Papi and Tongiorgi, 1963; Williamson, 1951). In this paper we attempt to ascertain the cues that are utilized in underwater orientation by *Uca pugilator* and any hierarchial arrangement of these cues.

MATERIALS AND METHODS

Most of the experiments were conducted at Orient Beach State Park, Long Island, New York, during July, August, and September, 1972, and the Mote Marine Laboratory, Placida, Florida, in January, February, and March, 1973. Four experiments were performed with Florida crabs at Ithaca, New York.

The crabs were tested in two tall narrow tanks made of clear 0.6 cm levate (Plexiglas); the interior dimensions were 77.6 cm long, 12.5 cm wide, and 51.5 cm high. Since the focus of this study was only upon whether the crabs oriented landward or seaward, the absolute compass bearing of the released crabs was inconsequential. Accordingly, the narrow or "one-dimensional" characteristic of the testing tanks provided the subjects with essentially only two directions in which to travel. An open-topped rectangular tank served as the standard testing apparatus, and a trapezoidal tank was used in the gradient experiments to be discussed later.

At the bottom of each testing tank was a levate ramp, with an adjustable angle of inclination, in which 3 mm wide "foothold" grooves had been cut. Test crabs were introduced from above through a 2.5 cm ID (internal diameter) levate tube, 63 cm in length. The experimenter, standing 6 m away on a line perpendicularly bisecting the long axis of the tank, could release a crab by removing a wire platform located beneath the crab and 10 cm from the top of the tube. A small lead sinker above the crab forced the crab onto the middle of five contiguous, congruent zones (each 15.2 × 12.1 cm) marked on the ramp.

Generally, the testing apparatus was submerged in 20 to 30 cm of water directly offshore and perpendicular to the crabs' home beach. The home burrows were within 15 m of the tank. The water level inside the tank was maintained within 4 cm of that of the surrounding water. To minimize the possible accumulation of metabolites within the tank, the water was changed after every 6 to 7 crab releases using local sea water. Since above-water orientational responses vary

Table I

Experimental results of control and isolated cue experiments, comparing the number of crabs orienting in one direction against the number orienting in the opposite direction. Asterisks denote significance at the 0.05 level. Approximate times of day are given for those tests involving celestial cues.

Experiment	Location	Month	Approx. time	Number of crabs orienting toward:		Significance level (two-tailed)
				Home beach	Deep water	
Control I "natural" conditions	Florida	Jan. 73	1500–1700	16	1	$P < 0.001$*
		Feb. 73	1130–1300	15	0	$P < 0.001$*
		Mar. 73	1430–1615	17	0	$P < 0.001$*
	Long Island	Jul. 72	1645–1845	17	2	$P < 0.005$*
		Sep. 72	1430–1730	22	10	$P > 0.05$
Control II total overcast landmarks obscured tank level	Florida	Jan. 73		10	9	$P > 0.95$
	Long Island	Aug. 72		10	9	$P > 0.95$
Celestial cues only	Florida	Jan. 73	1330–1700	15	1	$P < 0.005$*
	Long Island	Aug. 72	1230–1445	14	4	$P < 0.05$
Landmark cues only	Florida	Jan. 73		20	4	$P < 0.005$*
	Long Island	Aug. 72		17	13	$P > 0.5$
Gradient cues only	Florida	Feb. 73		20	9	$P > 0.05$
		Aug. 73		15	5	$P < 0.05$*
		Aug. 73		18	7	$P < 0.05$*

among crabs of different size and age categories (Herrnkind, 1972), only typically colored male and female adults with carapace width greater than 10 mm were used. They were captured and tested one at a time during daylight hours of normal feeding and/or social activity. Once captured, the test crab was transported in a closed fist along the straightest line to the experimental tank. After a crab was released, the time it spent in each zone was recorded and its position noted after two minutes. Each individual was tested only once, marked, and then returned to the capture site. Marked crabs were not used in subsequent tests.

The following experimental manipulations were executed in order to determine the factors involved in landward orientation.

Controls

Control I. To ensure that crabs could orient landward while submerged in the testing tank, experiments were periodically conducted under "natural" conditions. The skies were clear and sunny, beach landmarks were available, and the testing tank was placed in the water on the natural slope of the subtidal and intertidal zones. The dates of the experiments are presented in Table I.

Control II. If the crabs could orient in the absence of celestial, landmark, and gradient cues, then they would have to be utilizing some aspect of the environment that we had not yet considered. Experiments to test this hypothesis were performed on heavily overcast days when sun and blue skies were not visible to human observers. The testing tank was levelled on the substrate in order to eliminate possible gradient cues. Landmarks were obscured by surrounding the tank with a screen constructed of four layers of translucent polyethylene sheeting (0.02 cm in thickness) stretched around eight poles. The resulting octagonal arena measured 0.97 m high and approximately 2.5 m in diameter.

Isolation of cue systems

Each of the three cue systems was isolated by eliminating the other two in the following manner.

Celestial cues. Experiments were conducted under clear, sunny skies with the tank levelled and the landmark-obscuring screen in place.

Landmark cues. The tank was levelled and the experiments were performed on days of total overcast.

Gradient cues. The tank was set on the natural slope of their home shore (3°–5°) with the landmarks obscured and the skies totally overcast. The infrequency of overcast days in southwestern Florida, however, forced collection of a large majority of these data under modified conditions. The testing tank was submerged in a circular plastic wading pool, 1 m in diameter, located in a radially symmetrical tent lined with black plastic to eliminate celestial and landmark cues. A 9° inclination was created *via* the adjustable ramp. The crabs could not be collected and tested individually since it was not possible to place the tank, pool, and tent within reasonable distance of their burrows. Consequently, they were collected in lots of eight to ten, transported in a light-proof container, and tested within 90 minutes of capture.

Conflict of cue systems

Pairs of cues that both normally indicated the direction of the home beach and that were shown to be used by crabs were conflicted to give opposing directional information. The resultant behavior in this conflict situation was assumed to be indicative of the dominance (if any) of one stimulus over the other. Pairs of different cue systems were conflicted as follows.

Gradient cues vs. celestial cues. Crabs were tested with the sun shining brightly, the landmarks obscured, and the gradients reversed. In other words, the deeper end of the tank was closest to the home beach. Subjects that moved toward deeper water would be orienting according to celestial cues, and thus reveal that celestial cues dominate gradient cues. Crabs that oriented toward the shallow end of the tank would indicate preferences for gradient cues over celestial cues.

Landmark cues vs. celestial cues. In order to conflict landmark and celestial cues, the entire experimental apparatus was transported 0.7 km to an opposing but parallel beach on a small "spoil" island on the west side of the Intra-Coastal Waterway. The short pines, mangroves, and beach grasses of this beach resembled the vegetation of the home beach. Twenty crabs were collected on the home beach, transported in lightproof containers to the opposing beach, and tested individually within 75 minutes of capture.

Gradient cues vs. landmark cues. On a totally overcast day, gradient cues were conflicted with landmark cues by sloping the tank upward, away from the landmarks on the home beach.

Gradient cues

The gradient cue most obvious to a crab on the shore is presumably the substrate slope; underwater movements, however, could involve gradients of light intensity, light wavelength, and pressure, as well as the substrate slope. Temperature and chemical gradients were not likely to exist in the region of shore that we were considering due to constant mixing. Once it was established that, in fact, the crabs did respond to gradient cues in the absence of landmark and celestial cues, the next problem was to determine which gradient cues were utilized and how they were related.

For this series of experiments, a tank with a floor similar to the rectangular tank but with trapezoidal sides, was constructed of 6 mm clear levate. A sagittal view of this tank resembled a right triangle that was truncated and opened at its upper acute corner. During all of the following experiments, this tank was levelled and submerged in 10 cm of water in the wading pool within the tent. The crabs were collected in lots of ten, held in a lightproof container, and tested individually within 90 minutes of capture. The August, 1973 experiments were conducted in Ithaca, New York, with crabs from Clearwater, Florida. They were transported by car and testing began within 48 hours of capture. While in Ithaca, the crabs were "housed" on artificial beaches under the same tidal regime and photoperiod as those of their home beach.

Light intensity and light wavelength. All vertical surfaces of the tank were covered with black polyethylene, permitting light to reach the bottom of the tank only through the slanted top surface. Two fluorescent bulbs [producing 75 footcandles (1.19 watts m^2) at one foot and having a correlated color temperature of 4300° and a color rendering index of 69] were positioned 1.2 m above and parallel to the tank bottom. While these lamps may not accurately mimic sunlight, light intensity and light wavelength gradients were nevertheless established along the tank bottom in the absence of any substrate slope or changing water pressure. The hydrostatic pressure at every point along the tank bottom was equal to the highest column of water (51 cm). Each crab was released into the central region of the light gradient by means of an introduction tube, like that used in the rectangular tank. A crab travelling toward the "short" end of the tank encountered photic conditions resembling those met during movements from deep water to shallow water. The covered sides of the tank created a problem; once a crab was released, its movements could not be monitored without disturbing the crab or destroying the light gradient. Consequently, for 2 minutes after it was released, the test subject was left undisturbed. At the end of that period the experimenter peered over the top of the tank and noted the crab's position along the bottom. Any movement after that point might have been a fright response and was not included in the analysis.

Hydrostatic pressure. Creating a hydrostatic pressure gradient while maintaining a level substrate proved to be impossible. However, by servoing the pressure at the tank bottom to the position of the crab, a pressure gradient could be simulated while eliminating gravitational and photic cues. As the crab moved toward one end of the tank, the pressure within the tank was gradually increased; if it moved in the opposite direction the pressure was correspondingly decreased.

The trapezoidal tank was modified for this pressure gradient experiment. An opaque gasket-lined cover was fitted onto the opening at the "tall" end of the tank. Leading through this cover was 15 cm of flexible 1.9 cm ID tubing. A "pressure tube" of rigid Plexiglas (63 cm long, 2.5 cm ID) was connected to the distal end of the flexible tubing. To eliminate light cues all tank surfaces, with one exception, were covered with black polyethylene. The lower 8 cm along one side was left open to light to enable the experimenter to monitor the test subject's position and adjust the pressure accordingly. The maintenance of positive pressure within the tank necessitated the sealing of the crab introduction tube. Each trial began by placing the crab in the tube on a hinged metal platform that remained horizontal as long as a magnet was placed on the outside of the tube. The introduction tube was then filled with sea water and sealed with a rubber stopper. Next, the pressure tube was held at an angle of approximately 3° above horizontal, filled with sea water, and left open to the air. As this tube was raised to a vertical position the height of the column of water within the tube rose, gradually increasing the pressure within the tank. The maximum pressure change that could be created at the tank bottom was equivalent to a simulated slope of 12°. With the pressure set midway between the maximum and minimum levels, the magnet was removed from the outside of the introduction tube, and the test crab dropped down to the tank bottom. The hydrostatic pressure within the tank was then manually servoed to the movements of the crab for two minutes, using the full range of pressure (53–69 cm of water).

Light intensity, light wavelength, and pressure. Only minor changes in the pressure gradient set-up were required in order to create a combination of light intensity, light wavelength, and pressure gradients in the absence of substrate slope. First, the black plastic covering the top slated surface of the trapezoidal tank was removed to supply the light gradients. Next, to prevent light from entering the window through which the crab's movements were monitored, an "awning" of black cardboard was erected above the observation window. Each crab was then tested in the same fashion as described in the pressure gradient experiment.

The analysis of the data from the Long Island and Florida crabs was divided into three phases. First, in each experiment dealing with different cues regimes, the number of crabs orienting in one direction was compared to the number orienting in the opposite direction. Next, the proportions of shoreward orienting crabs from different experiments were compared. Finally, the times required for orientation in the different experiments were compared. Each crab's orientation time was the number of seconds taken to finally enter the zone in which it remained until the end of the trial. Hence, a crab that wandered continuously would have an orientation time close to 120 seconds; an orientation time of 5 seconds would characterize a crab that headed toward one end of the tank upon release and remained in that end zone until the termination of the trial. Any crab found in the central three zones after two minutes was considered to have not oriented and was not considered in this analysis.

The numbers of crabs orienting in opposite directions within a given experiment were compared using a Chi-square test, corrected for continuity (Snedecor and Cochran, 1967). [These results are presented in Table I and Table II.]

TABLE II

Experimental results of conflicted cues and gradient aspects, comparing the number of crabs orienting in one direction against the number orienting in the opposite direction. Asterisks denote significance at the 0.05 level. Approximate times of day are given for those tests involving celestial cues.

Experiment	Location	Month	Approx. time	Number of crabs orienting according to:		Significance level (two-tailed)
				Gradients	Celestial cues	
Gradient *vs.* celestial cues	Florida	Jan. 73	1200–1515	5	17	$P<0.025$*
	Long Island	Aug. 72	1250–1500	5	12	$P>0.1$
				Landmarks	Celestial Cues	
Landmarks *vs.* celestial cues	Florida	Feb. 73	1300–1415	4	15	$P<0.025$*
				Gradients	Landmarks	
Gradient *vs.* landmarks	Florida	Mar. 73		7	10	$P>0.5$
				Number of crabs orienting toward:		
				Shallow water	Deep water	
Light intensity and light wavelength gradients	Florida	Mar. 73		8	12	$P>0.5$
Pressure gradient	Florida	Aug. 73		14	10	$P>0.5$
Light intensity, light wavelength and pressure gradients	Florida	Aug. 73		9	8	$P>0.9$

The proportions of shoreward orienting crabs from various experiments were compared using a 2 × 2 contingency table and either the Chi-square test, corrected for continuity, or the Fisher exact probability test, depending on the size of the sample (Siegel, 1956).

RESULTS

Controls

Florida crabs consistently oriented toward their home beach under "natural" conditions when celestial, landmark, and gradient cues were available (Control I) (Table I). No temporal changes in the crabs' ability to orient toward shore were noted, since one experiment was performed at the beginning of the Florida series of experiments (January, 1973), one in the middle (February, 1973), and one at the end (March, 1973). The Long Island crabs also displayed orientation toward shore, but with more ambiguity than their southern counterparts. The results of the July experiment were highly significant ($P < 0.005$; Table I). The results of the September experiment yielded a nonsignificant χ^2 of 3.78 (χ^2 critical, $\alpha = 0.05$, with 1 d.f. $= 3.84$).

A comparison between the proportions of Long Island and Florida populations that oriented under "natural" conditions revealed that Florida crabs oriented toward shore more frequently than did Long Island crabs ($P < 0.05$; Table III).

TABLE III

Comparisons of the proportions of shoreward orienting crabs from pairs of selected experiments. Asterisks denote significance at the 0.05 level.

Experiment	Location	Number of crabs orienting toward: Home beach	Number of crabs orienting toward: Deep water	Test	Significance level (one-tailed)
Control I	Florida Long Island	48 39	1 12	χ^2	$P < 0.005$*
Celestial only Landmarks only	Florida	15 20	1 4	χ^2	$P > 0.25$
Celestial only Gradients only	Florida	15 53	1 21	χ^2	$P < 0.05$*
Landmarks only Gradients only	Florida	20 53	4 21	χ^2	$P > 0.1$
Celestial only Gradients *vs.* celestial	Florida	15 17 (cel.)	1 5 (grad.)	Fisher	$P > 0.1$
Celestial only Landmarks *vs.* celestial	Florida	15 15 (cel.)	1 4 (land.)	Fisher	$P > 0.2$

When neither celestial, landmark, nor gradient cues were present (Control II) both Long Island and Florida crabs distributed themselves evenly toward and away from shore (Table I). Thus, it is unlikely that magnetic fields, or other visual cues influenced the orientation of the crabs under these test conditions. While the nature of the testing apparatus isolated the test crabs from the potential influence of wave surge and nearshore water currents, hydrodynamic cues are often absent in the shallow, protected estuaries characteristic of the habitat of *U. pugilator.*

Isolated cues

When only celestial cues were present, both the Florida and Long Island groups of crabs were able to direct themselves toward shore ($P < 0.005$ and $P < 0.05$, respectively). However, with only landmark cues available, the Florida crabs displayed significant shoreward orientation ($P < 0.005$), while the Long Island crabs did not ($P > 0.05$) (Table I).

Results of tests on three different groups of Florida crabs presented with only gradient cues are shown in Table I. Two of the three experiments resulted in significant landward orientation, while the remaining experiment yielded a non-significant χ^2 of 3.44 (χ^2 critical, $\alpha = 0.05$, with 1 d.f. $= 3.84$). Long Island crabs were not tested for their ability to use gradient cues alone due to the lack of appropriate facilities there.

The proportion of Florida crabs that oriented shoreward when only celestial cues were available was significantly greater than the proportion that used only gradient cues ($P < 0.05$). No such difference was apparent between celestial and landmark cues, nor between landmark and gradient cues (Table III).

Conflict of cue systems

When landmarks were eliminated and gradient cues were conflicted with celestial cues, there was no significant orientation among Long Island crabs that was consistent with either cue system. Among the Florida crabs, a significantly larger number oriented with the celestial cues than with the gradient cues ($P < 0.025$) (Table II). Nevertheless, there was no significant difference between the results of this gradients *vs.* celestial cues experiment and those of the isolated celestial cues experiment. In other words, the presence of the opposing gradient cues did not significantly affect the proportion of the crabs that normally respond to celestial cues alone (Table III).

The conflict between landmarks and celestial cues, in the absence of any gradient cues, results in a marked dominance of celestial cues ($P < 0.025$) (Table II). Again, the presence of opposing landmarks did not significantly affect the proportion of crabs that oriented according to celestial cues alone (Table III). The dominance of celestial cues over landmarks might be more convincing had the landmarks on the "spoil" island been shown to be effective guidance cues in the absence of gradient and celestial cues.

When gradients and landmarks were presented in opposing fashion, neither cue system displayed a clear dominance over the other (Table II).

Gradient cues

Having established that *Uca pugilator* is capable of using some gradient(s) for shoreward orientation (Table I), it was necessary to determine which aspect(s) of a water depth gradient the crabs were using. A combination gradient of light intensity and light wavelength did not result in any significant orientation of the crabs (Table II). Likewise, a simulated hydrostatic pressure gradient appeared to be an ineffective stimulus (Table II). When the crabs were subjected to a combined gradient of pressure, light intensity, and light wavelength no significant response resulted; approximately half the crabs travelled toward deep water and half toward shallow water (Table II). It is highly unlikely, however, that these crabs at this point had lost their ability to orient with gradient cues. As a test, at the next low tide another batch of crabs from the same beach was presented with all of the gradient cues; light intensity, light wavelength, hydrostatic pressure, and substrate slope. The outcome, previously presented, shows significant shoreward orientation (18 crabs oriented toward shallow water and 7 toward deep water) ($P < 0.05$) (Table I).

In all cases where significant orientation occurred under the same experimental conditions in both Florida and Long Island crabs, statistical comparisons of the orientation times between the two sites were made. Under "natural" conditions, it was found that Florida crabs ($\bar{X} = 21.0$ sec, $N = 44$) oriented sooner after release than Long Island crabs ($\bar{X} = 39.1$ sec, $N = 22$) ($P < 0.05$, Table IV). No significant difference existed between the orientation times of Florida and Long Island crabs when both groups were exposed only to celestial cues (Table IV).

Florida crabs that were presented with either isolated celestial cues, landmarks, or gradients did not significantly differ in their orientation times. For instance,

Table IV

Comparisons of the orientation times from selected experiments. Asterisks denote significance at the 0.05 level.

Experiment	Location	$\bar{X}$, sec	N	Test	Significance level (one-tailed)
Control I	Florida	21.0	44	Mann-Whitney U	$P < 0.05$*
	Long Island	39.1	22		
Celestial only	Florida	19.0	14	Mann-Whitney U	$P > 0.1$
	Long Island	30.9	11		
Celestial only	Florida	19.0	14	Kruskal-Wallis	$P > 0.1$
Landmarks only	Florida	40.7	18		
Gradients only	Florida	56.8	50		
Gradients *vs.* landmarks					
Gradients	Florida	48.5	4	Mann-Whitney U	$P > 0.1$
Landmarks		17.2	6		

celestial cues did not result in significantly faster orientation than either landmarks or gradients (Table IV).

Discussion

The terrestrial orientation of *Uca pugilator* has been investigated by Herrnkind (1972). On aerially exposed substrates, landmarks and celestial cues, in the form of sun position and polarized light, are utilized for directional guidance. Celestial cues generally override landmark cues in cases where they conflict. Kinesthetic cues are used only in the immediate vicinity of the burrows. Among the species studied so far, gravitational cues act as guideposts in the wolf spider *Arctosa* (Herrnkind, 1972), the isopod *Tylos punctatus* (Hamner, Smyth, and Mulford, 1968), and the amphipod *Orchestroidea corniculata* (Craig, 1973).

Underwater orientation in *Uca pugilator* involves the use of gradient cues, as well as landmark and celestial cues. Each cue system alone would permit a crab to emerge from the water (Table I). While celestial cues appear to be more influential than landmark and gradient cues (Table II), the relative importance of the latter two cue systems is not as obvious. Neither proved to be the dominant mode when they were conflicted (Table II). It should be noted, however, that the one-dimensional character of the test tanks obscures any response having an angular component relative to the axis of the tank. When landmarks were conflicted with gradients, the time the crabs took to orient according to either system did not significantly differ (Table IV). It must be noted, though, that a type II error is quite likely in this case due to the small sample size ($n_1 = 4$, $n_2 = 6$). If the respective orientation times were different, perhaps a more subtle form of dominance was then occurring, such as rate of orientation.

Landmark cues take the form of differential light intensities along the horizon. Celestial cues are potentially available to the underwater crab in three forms: the sun's position, the polarized light of blue sky (as in the terrestrial case), and the polarized daylight originating from the water itself (Waterman, 1954). Since the latter two are ultimately dependent upon the sun's position, little is gained from attempts to conflict assorted pairs of the trio.

Gradient cues exist as light intensity, light wavelength, and hydrostatic pressure gradients, as well as substrate slope. The results here tend to reject the hypothesis that *Uca pugilator* employs any combination of the first three gradients for shoreward orientation. Barosensitivity among brachyurans is generally limited to the planktonic larval stage (Baylor and Smith, 1957; Digby, 1967; Hardy and Bainbridge, 1951; Knight-Jones and Qasim, 1965; Morgan, 1972). To date, pressure sensitivity among adult brachyurans has been demonstrated only in the portunid crabs *Macropipus holsatus* (Morgan, 1967) and *Carcinus maenas* (Naylor and Atkinson, 1972). In both cases, increased pressure stimulated locomotor activity and the threshold of detection was less than 0.1 atm (or 1 m of water). Since the maximum pressure change induced by the trapezoidal tank was equivalent to only 16 cm of water, it is clear that the corresponding simulated slope of 12° over the length of the tank is substantially less than what true swimming crabs could detect without gravitational cues.

The remaining gradient cue to consider is the substrate slope. At the time these experiments were conducted, we had no feasible means of isolating substrate incline without producing pressure gradient at the same time. However, positive pressure inside a sealed container exerts itself equally and undiminished on all sides (Pascal's Principle). Thus, a crab inside a pressurized rectangular tank, mounted on an incline, will experience a substrate slope in the absence of any pressure gradients. Although this experiment was not conducted, these crabs are morphologically capable of detecting gravitational cues associated with the substrate slope. Typical brachyuran statocysts are located at the bases of the crabs' antennules (Schone, 1971). In addition, their limb proprioceptors can monitor the movement, stress, and position of their ambulatories (Cohen and Dijkgraaf, 1961). This evidence, coupled with the fact that all gradient cues other than substrate slope have been experimentally excluded, leads us to believe that the aspect of gradient to which *Uca pugilator* responds is the gravitational cue that is associated with substrate slope.

Generalizations cannot be made concerning the behavioral differences between Florida and Long Island crabs since the samples tested were only from two beaches. Further testing on other Gulf and Atlantic coast beaches would be needed to detect any true differences in the proportions of shoreward orienting crabs, or any clines of orientation time. Potential differences might be due to the genetic composition of local populations or to environmental factors such as local pollution levels (J. Lincer, Mote Marine Laboratory, personal communication).

Uca pugilator has the ability to orient both on land and underwater. The terrestrial and underwater systems are similar in that they employ redundant schemes of cues, with celestial cues dominating. By inference, this study has shown that, in addition, substrate slope may be used for directional guidance underwater. Its role in above-water orientation, however, has yet to be demonstrated.

We thank the Long Island State Park Commission for their cooperation during the Long Island phase of this project, and the Mote Marine Laboratory, Dr. Perry Gilbert, Dr. Oliver Hewitt, and Mr. Stewart Springer for their generous assistance in Florida. Drs. Stephen T. Emlen, Michael Salmon, and William Herrnkind read the manuscript, and we appreciate their helpful comments and suggestions. Dr. Melvin Kreithen's input concerning fluid mechanics is gratefully acknowledged.

SUMMARY

The semi-terrestrial sand fiddler crab, *Uca pugilator,* occasionally is forced by avian and mammalian predators to go into the water where it becomes vulnerable to aquatic predators. Therefore, it would be adaptive for *Uca* to possess some means of detecting the direction of the shore and its burrow while submerged. Using crabs from Florida and Long Island, New York, the identity of the cues used in underwater orientation and the possible hierarchial arrangement of these cues were ascertained.

Under various cue regimes, the crabs were individually observed in a long narrow tank that allowed the crabs to proceed either toward shore or away from it. The potential cues for shoreward orientation which were available to the submerged crab were celestial cues, landmarks, and gradient cues (hydrostatic pressure, light wavelength, light intensity and substrate slope). Each cue was isolated and then tested for its effectiveness in orientation. It was next presented to the crabs in a conflicting configuration with other cues to determine its relative effectiveness.

The crabs oriented toward shore when presented with either celestial cues, landmarks, or gradient cues. Celestial cues dominated both landmarks and gradient cues, while no clear dominance was observed between landmarks and gradient cues. Gradients of hydrostatic pressure, light wavelength, and/or light intensity were found to be ineffective cues for orientation. This suggests that the gradient cue used for shoreward orientation is the substrate slope. The behavioral differences between Florida and Long Island crabs were also examined.

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Volume 155 Number 2

Marine Biological Laboratory
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THE
BIOLOGICAL BULLETIN

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OCTOBER, 1978

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $8.00. Subscription per volume (three issues), $22.00, (this is $44.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

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4. ***Literature Cited.*** The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of THE BIOLOGICAL BULLETIN, and must be typed *double-spaced* on separate pages. Note that citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list

Continued on Cover Three

Vol. 155, No. 2 October, 1978

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

SEASONAL PATTERNS IN THE BIOCHEMICAL CONSTITUENTS AND BODY COMPONENT INDEXES OF THE MURICID GASTROPOD, *THAIS HAEMASTOMA*

Reference: *Biol. Bull.*, **155**: 259–272. (October, 1978)

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There are several methods currently employed in determining reproductive cycles in marine invertebrates. One of the most useful to date is that of determining body component/body weight ratios. These ratios, known collectively as body component indexes, allow for an overall view of the entire reproductive state of an organism and, coupled with determination of various biochemical constituents (*i.e.*, lipid, carbohydrate and protein), enable one to trace the accumulation and mobilization of organic reserves associated with annual reproductive cycles. Other methods of studying the reproductive states of marine invertebrates have been summarized by Giese (1959) and include spawning, numbers of larvae, appearance of ripe gametes, brooding of eggs and relative size of gonads.

The reproductive cycles of a variety of temperate species of prosobranch gastropods have been established. Houston (1971) utilized histological and histochemical techniques in studying the reproductive biology of two West Coast species, *Thais canaliculata* and *T. emarginata*. Seasonal cycles in *T. lamellosa* have been determined using the index method (Stickle, 1973; Lambert and Dehnel, 1974). Other prosobranchs which have been studied include *Fusitriton oregonensis* by Stickle and Mrozek (1973), *Haliotis cracheroidii* by Webber (1970) and Webber and Giese (1969) and *Littorina littorea* by Williams (1970).

The capsule-albumin gland index has been determined to be the best component indicator of reproductive readiness in several species of prosobranchs, including *F. oregonensis* (Stickle and Mrozek, 1973) and *T. lamellosa* (Stickle, 1973). In a subtropical species such as *Thais haemastoma*, where spawning is intermittently interspersed with feeding, short term fluctuations in the body component indexes may be considerable.

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Energy requirements during the reproductive period of an organism may be relatively expensive. For example, 30% of the prespawning soft body weight of *F. oregonensis* was lost during aggregation and capsule deposition (Stickle and Mrozek, 1973), while 34% was lost during the same period by *T. lamellosa* (Stickle, 1973). Many animals meet the metabolic expense of spawning by drawing on reserve materials accumulated during a prespawning refractory period. Seasonal changes in body component indexes have been shown to reflect fluctuations in biochemical constituents in several species of marine gastropods. Stickle (1975) found seasonal changes in the protein, carbohydrate and lipid content of body components in *T. lamellosa* to be due predominantly to changes in the component indexes and not to fluctuations in constituents levels. Bistransin (1976) found similar results in the subtropical *Littorina irrorata*.

These analyses were performed to determine seasonal patterns in the body component indexes and biochemical content and level of lipid, carbohydrate and protein in the subtropical prosobranch gastropod, *T. haemastoma*.

MATERIALS AND METHODS

Body component indexes

Adult specimens (>30 mm) of *Thais haemastoma* were collected at approximately monthly intervals from September, 1973, through December, 1974. Animals were obtained from the concrete pilings supporting the bridge spanning Caminada Pass, Louisiana. Water temperature and salinity were determined at each collection. Live animals were transported to Louisiana State University where they were placed in collection jars or plastic containers and stored at −20° C. Dissections were begun in the fall of 1975 and continued through the spring of 1977.

Animals were removed from the freezer and encrustations scraped from the shell. Shell length, taken as the distance from the apex to the tip of the siphonal canal, was determined with a vernier caliper. The shell and foot were blotted dry and the entire animal including the shell and operculum was weighed. This weight was designated as the entire weight. When animals were completely thawed the shell was cracked and the operculum and all shell pieces removed. The animal was immediately dissected into body components and the total wet weight determined. The shell weight was taken as the entire weight minus the soft body wet weight. Male body components consisted of shell, body water, foot, testis-digestive gland (TDG), and remaining visceral mass (RVM). Female body components consisted of shell, body water, foot, ovary-digestive gland (ODG), remaining visceral mass (RVM) and capsule-albumin gland (CAG). Individual components were flash frozen between two cakes of dry ice, lyophilized to dryness and placed in a desiccator. Dried components were weighed to the nearest mg, and this weight was designated as the component dry weight. Lyophilized components were stored at −20° C. Individual body component indexes were calculated according to the method of Stickle (1973).

Biochemical analyses

Lyophilized body components were removed from the freezer and warmed to room temperature in a dessicator. Individual components were ground in a

Thomas-Wiley tissue grinder and stored at −20° C in one dram glass vials with cork stoppers. Protein and carbohydrate were extracted by refluxing 10 mg of ground tissue in 20% KOH at 100° C. The digest was diluted to yield a final KOH concentration of 1%. Aliquots of this dilution were used in all subsequent protein and carbohydrate analyses. Protein was determined using a modified Gornall biuret test (Gornall, Bardawill and David, 1949). Samples were corrected for turbidity and color interference by subtracting a specific minus copper reference tube from each tube reading. Bovine serum albumin was used as a reference standard. Total carbohydrate was determined by the method of Dubois, Gilles, Hamilton, Rebers and Smith (1956) using glycogen as a reference standard.

Lipids were extracted from dry, ground tissue using a methanol: chloroform: water (2: 1: 0.8) procedure (Bligh and Dyer, 1959; Kates, 1972). Total lipids were determined gravimetrically.

Biochemical level and content

Total protein, carbohydrate and lipid level data are presented as percentage of dry weight. Biochemical content (total present per component of a 100 g standard animal) was determined by the method of Stickle (1975) where content equals the decimal fraction of level times the component index.

Statistical analyses

All variables are presented as the mean plus or minus the confidence interval at the ninety-five per cent level of significance. One way analysis of variance (ANOVA) was used to determine the significance of variation in soft body indexes and in biochemical content and level on a seasonal basis. The CAG index and the RVM, ODG, TDG and CAG biochemical content and level of males and females were further studied using Duncan's Multiple Range Test. "Foot" data was not analyzed with the Duncan's Test because variations in that component are minor.

Shell weight–shell length ratios were analyzed using the maximum r^2 improvement procedure of the dependent variable. Shell indexes were regressed on the mean shell weight–shell length ratios to determine whether or not index variations were due to fluctuations in the shell or in the soft body components. Differences were termed significant if $P < 0.05$ and highly significant if $P < 0.01$. All statistical analyses except the 95% confidence intervals were programmed and performed using a statistical analyses system (Barr, Goodnight, Sall and Helwig, 1976).

RESULTS

Body component indexes

The shell, body water and dry weight indexes and collection data for males and females are found in Table I. Shell weight/shell length ratios exhibited significant variations with time; however, linear regression analysis of monthly mean shell indexes on mean shell weight/shell length ratios over the course of the study indicates there was no significant relationship between these two variables in either sex. Body water and dry weight indexes cycled inversely to the shell index.

TABLE I

Collection data, shell, body water and dry weight indexes for male and female Thais haemastoma. *Indexes are presented as the mean plus and minus the ninety-five per cent confidence limit. N.D. indicates no data.*

Date	Water temp. (°C)	Salinity (‰)	Sample ♂	Size ♀	Shell index		Body water Index		Dry weight Index	
					♂	♀	♂	♀	♂	♀
Sept. 1973	30.5	18.0	14	9	77.35 ± 2.09	78.73 ± 2.87	16.64 ± 0.47	15.72 ± 2.17	6.01 ± 0.68	5.55 ± 0.80
Oct. 1973	25.2	22.0	11	5	73.79 ± 1.22	76.13 ± 2.81	19.60 ± 1.13	15.85 ± 1.78	6.59 ± 0.80	6.02 ± 1.82
Dec. 1973	13.8	27.0	5	8	80.64 ± 1.94	80.57 ± 3.30	13.00 ± 1.78	12.66 ± 2.13	6.36 ± 0.69	5.63 ± 0.76
Jan. 1974	10.0	19.0	13	9	78.00 ± 1.38	82.55 ± 2.74	15.58 ± 0.91	13.05 ± 1.63	6.55 ± 0.79	4.40 ± 1.07
Feb. 1974	20.0	12.0	13	9	83.79 ± 1.03	83.75 ± 1.18	12.25 ± 0.87	12.03 ± 0.94	3.98 ± 0.33	4.22 ± 0.40
March 1974	21.5	11.0	17	7	84.15 ± 1.23	84.00 ± 2.76	11.93 ± 0.08	12.03 ± 1.58	3.92 ± 0.49	3.53 ± 0.74
April 1974	24.9	16.0	12	12	76.33 ± 1.56	77.43 ± 1.62	17.32 ± 0.94	15.82 ± 0.96	6.36 ± 0.76	6.75 ± 0.73
May 1974	N.D.	N.D.	20	14	80.68 ± 1.15	81.95 ± 1.11	14.45 ± 0.75	13.18 ± 0.82	4.88 ± 0.45	4.87 ± 0.42
June 1974	29.0	23.5	24	9	82.21 ± 0.78	81.83 ± 1.98	13.20 ± 0.61	13.07 ± 1.32	4.58 ± 0.21	5.11 ± 0.73
July 1974	32.5	19.0	19	9	84.31 ± 1.41	83.31 ± 2.43	10.90 ± 0.90	11.34 ± 1.62	4.59 ± 0.51	4.88 ± 0.81
Aug. 1974	22.8	20.0	21	8	82.71 ± 1.62	83.71 ± 2.64	12.60 ± 1.11	11.28 ± 1.76	4.69 ± 0.57	5.00 ± 1.02
Sept. 1974	27.8	20.5	13	13	80.71 ± 2.16	80.80 ± 1.69	13.94 ± 1.34	13.92 ± 1.00	5.36 ± 0.93	5.28 ± 0.73
Oct. 1974	22.5	28.0	10	6	81.21 ± 1.55	84.67 ± 1.76	13.60 ± 1.13	11.01 ± 1.13	5.50 ± 0.78	3.99 ± 1.30
Nov. 1974	19.0	30.5	10	10	84.92 ± 1.83	85.98 ± 1.26	11.35 ± 1.15	10.72 ± 0.94	3.72 ± 0.84	3.30 ± 0.74
Dec. 1974	11.5	23.5	7	1	84.64 ± 1.73	85.49	11.31 ± 1.57	10.74	4.05 ± 0.24	3.77

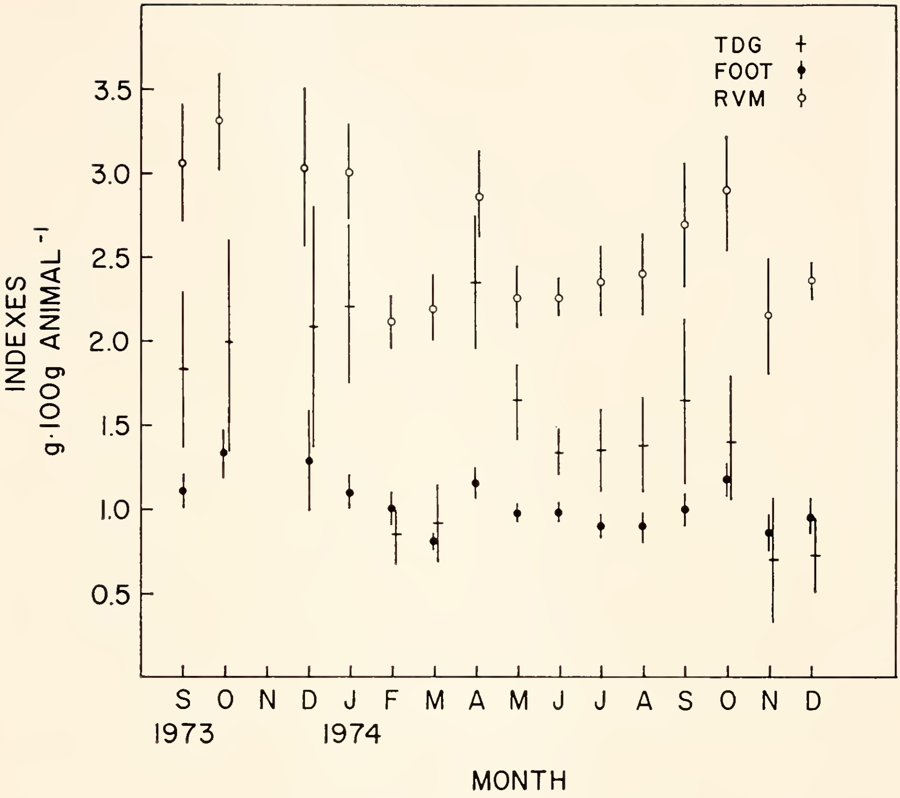

FIGURE 1. Body component indexes in *Thais haemastoma.* Male testis-digestive gland (TDG), remaining visceral mass (RVM) and "foot" indexes are presented as the mean (symbol) plus and minus the ninety-five per cent confidence interval (vertical line).

Linear regression analysis of percentage of body water on salinity indicates there was a significant inverse relationship ($n = 190$, $r^2 = 0.03$, $P < 0.05$) between these two variables in males, but no significant relationship existed in females. Analysis of dry weight on temperature does not show a significant relationship for males while a significant positive relationship ($n = 114$, $r^2 = 0.05$, $P < 0.05$) occurred in females.

Male RVM, foot and TDG indexes are found in Figure 1. One way analysis of variance (ANOVA) indicates that all three components showed highly significant variations ($P < 0.01$) with time. However, seasonal changes in the magnitude of the foot indexes were small when compared with changes in the magnitude of the RVM and TDG indexes. There appear to be bimodal patterns present in both the TDG and RVM indexes in 1974. The TDG index reached a primary peak of 2.35 g·100 g animal^{-1} in April, 1974 and a secondary peak of 1.65 g·100 g animal^{-1} in September, 1974. The RVM index peaked at 2.37 g·100 g animal^{-1} in April, 1974 and again at 2.91 g·100 g animal^{-1} in October, 1974. Indexes from April to September dropped to an intermediate value. Indexes in 1973 were generally higher than those observed in 1974, although sample variation within a month was greater.

Female RVM, foot and ODG indexes are presented in Figure 2. ANOVA indicates that all four components exhibited highly significant variations ($P < 0.01$) with time. However, seasonal changes in the magnitude of the foot and RVM indexes appear to be small when compared with seasonal changes in the magnitude of the ODG and CAG indexes. Seasonal patterns in the ODG are unimodal. This index peaked at 2.30 $g \cdot 100$ g animal^{-1} in April, 1974, declined during the summer months, and eventually reached a low of 0.46 $g \cdot 100$ g animal^{-1} in November. The CAG index peaked at 0.77 $g \cdot 100$ g animal^{-1} in April, 1974. This index dropped to intermediate values in May and June, 1974, showed a slight secondary peak in July at 0.56 $g \cdot 100$ g animal^{-1}, and dropped to a pre–peak low in August, 1974.

Male biochemical level

The mean protein level in males ranged seasonally from 62.07 to 80.24% dry weight in the foot, from 39.34 to 78.14% dry weight in the RVM, and from 23.91 to 61.78% dry weight in the TDG. All three components showed highly significant ($P < 0.01$) fluctuations in protein levels.

Mean carbohydrate level ranged from 6.37 to 11.32% dry weight in the foot, from 7.51 to 17.03% dry weight in the RVM, and from 6.18 to 10.00% dry weight in the TDG. Variations in the RVM and TDG carbohydrate level are highly significant ($P < 0.01$). Male and female monthly variations are discussed further under Duncan's Analysis.

Mean lipid level ranged seasonally from 4.05 to 4.97% dry weight in the foot, from 4.41 to 5.37% dry weight in the RVM and from 11.35 to 18.98% dry weight in the TDG. ANOVA indicates that variations in the RVM and TDG lipid levels are significant ($P < 0.05$).

Female biochemical level

Mean protein level in the females ranged seasonally from 59.63 to 74.58% dry weight in the foot, from 56.39 to 76.59% dry weight in the RVM, from 35.23 to 57.40% dry weight in the ODG and from 41.35 to 80.90% dry weight in the CAG.

Mean carbohydrate level ranged seasonally from 4.95 to 10.25% dry weight in the foot, from 7.79 to 18.85% dry weight in the RVM, from 5.15 to 10.22% dry weight in the ODG and from 4.21 to 14.18% dry weight in the CAG.

Mean lipid level ranged from 3.60 to 4.82% dry weight in the foot, from 4.18 to 5.54% dry weight in the RVM, from 12.26% to 19.55% dry weight in the ODG and from 4.17 to 13.48% dry weight in the CAG. ANOVA indicates that variations in protein, carbohydrate and lipid levels in all four female body components are highly significant ($P < 0.01$).

The highest mean protein level in both males and females was found in the foot and RVM, while the lowest mean level was found in the TDG and ODG. Mean carbohydrate level was generally higher in the RVM of both sexes. Mean lipid level was highest in the gonad digestive gland complexes and lowest in the foot in both sexes.

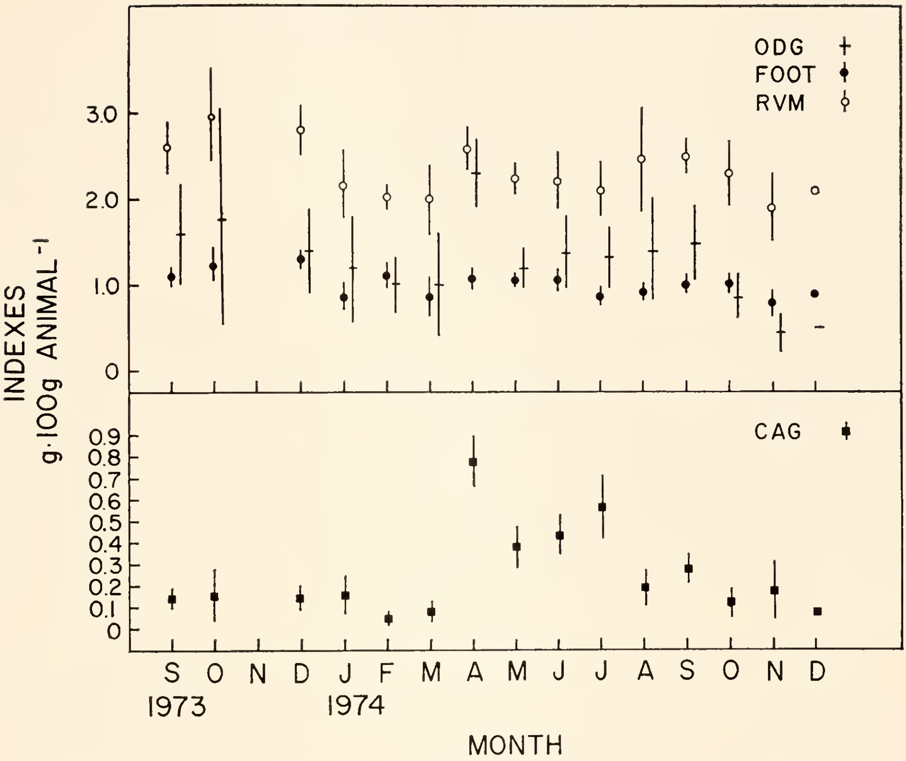

FIGURE 2. Body component indexes in *Thais haemastoma*. Female ovary-digestive gland (ODG), remaining visceral mass (RVM), capsule-albumin gland (CAG) and "foot" indexes are presented as the mean (symbol) plus and minus the ninety-five per cent confidence interval (vertical line).

Male biochemical content

Male protein content ranged from 0.53 to 0.85 g per 100 g animal in the foot and from 1.33 to 2.05 g per 100 g animal in the RVM. TDG protein content was 0.29 g per 100 g animal in February, increased to 1.04 g per 100 g animal in April and leveled gradually to a low of 0.18 g per 100 g animal in December. January TDG and RVM protein content appears to be high when compared with data obtained from other low temperature months of 1974. Variations in protein content in the RVM and TDG are highly significant ($P < 0.01$).

Mean carbohydrate content in the foot fluctuated from 0.06 to 0.13 g per 100 g animal. Mean RVM carbohydrate content increased significantly in April and June over other months. However, RVM carbohydrate content was highest in January with a value of 2.05 g per 100 g animal. TDG carbohydrate increased from 0.08 g per 100 g animal in February to 0.24 g per 100 g animal in April, declining gradually to 0.06 g per 100 g animal in November. ANOVA indicates that variations in RVM and TDG carbohydrate content are highly significant ($P < 0.01$).

Mean lipid content varied seasonally from 0.04 to 0.06 g per 100 g animal in the foot and from 0.10 to 0.15 g per 100 g animal in the RVM. January values for TDG lipid content appear to be abnormally high when compared with mean content values of other months. Mean TDG lipid content values were low in February at 0.17 g per 100 g animal and increased to a high of 0.49 g per 100 g animal in April. Lipid content dropped to 0.16 g per 100 g animal in June and then peaked again at 0.26 g per 100 g animal in September, returning to a low of 0.08 g per 100 g animal by December. ANOVA indicates that variations in mean lipid content in all three body components are highly significant ($P < 0.01$).

Female biochemical content

Mean protein content of females varied seasonally from 0.49 to 0.83 g per 100 g animal in the foot and from 1.15 to 1.77 g per 100 g animal in the RVM. Mean ODG protein content increased from 0.52 g per 100 g animal in March to 1.14 g per 100 g animal in April, dropped to 0.64 g per 100 g animal in May, rose slightly to 0.80 g per 100 g animal in June, and gradually decreased to its lowest value, 0.20 g per 100 g animal, in November. ANOVA indicates that variations in protein content in all three body components are highly significant ($P < 0.01$).

Mean carbohydrate content varied seasonally from 0.05 to 0.11 g per 100 g animal in the foot and from 0.18 to 0.48 g per 100 g animal in the RVM. Mean

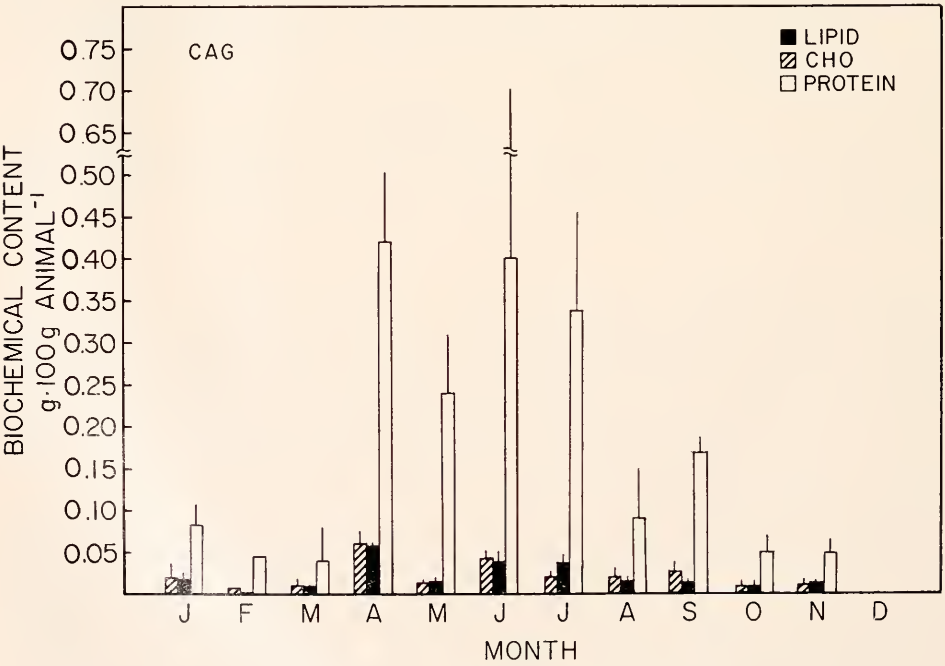

FIGURE 3. Biochemical content in *Thais haemastoma*. Protein, carbohydrate (CHO) and lipid content of the female capsule–albumin gland complex (CAG) expressed as the mean (vertical bar) plus the ninety-five per cent confidence interval (vertical line).

ODG carbohydrate content varied seasonally from 0.03 to 0.22 g per 100 g animal, with the largest increase occurring from 0.09 g per 100 g animal in March to 0.22 g per 100 g animal in April. ANOVA indicates that variations in carbohydrate content in all three body components are highly significant ($P < 0.01$).

Mean lipid content ranged seasonally from 0.04 to 0.05 g per 100 g animal in the foot and from 0.09 to 0.13 g per 100 g animal in the RVM. Mean ODG lipid content increased from 0.15 g per 100 g animal in March to 0.42 g per 100 g animal in April, but dropped to 0.24 g per 100 g animal in May and gradually decreased to a low of 0.07 g per 100 g animal in November. ANOVA indicates that fluctuations in foot and RVM lipid content are significant ($P < 0.05$) while fluctuations in ODG content are highly significant ($P < 0.01$).

Mean protein, carbohydrate and lipid content data in the capsule–albumin gland complex of females are found in Figure 3. All three CAG biochemical constituents showed highly significant fluctuations ($P < 0.01$). Protein content increased from 0.05 g per 100 g animal in March to 0.46 g per 100 g animal in April, but by November had fallen back to 0.05 g per 100 g animal. Carbohydrate content increased from 0.01 g per 100 g animal in March to 0.06 g per 100 g animal in April, and by November had fallen back to 0.01 g per 100 g animal. Mean lipid content increased from 0.01 g per 100 g animal in March to 0.06 g per 100 g animal in April and by November had again returned to 0.01 g per 100 g animal.

Duncan's analysis

Duncan's Multiple Range Test for the variable CAG indicates that this component index cycles in a significant bimodal pattern with peaks in April and July.

Duncan's analysis of male RVM level data indicates that carbohydrate level in this component cycles minimally. Carbohydrate level was highest in January and April and lowest in November. No other components in either males or females exhibited seasonal patterns in biochemical constituent levels.

The male TDG carbohydrate content exhibited a seasonal pattern of cycling. TDG carbohydrate content is significantly higher in April. TDG lipid also cycled significantly; lipid content was highest in April and January, although April was significantly higher than all other months while January was not. Lipid content in October was also significantly higher than November.

Male RVM carbohydrate content was significantly higher in January and April, and male RVM lipid content was significantly higher in April than in all other months.

Duncan's analysis of female biochemical content indicates that ODG protein cycles seasonally; protein content was significantly higher in April, June and January, although June is not statistically different from any other months except October and November. ODG lipid content also exhibited significant seasonal patterns. Lipid content was significantly higher in April than in all other months, while November content was significantly lower than all months except January, May and October.

Female RVM carbohydrate content is significantly higher in April from all months except June, while June is significantly higher than November, but is not different from any other months.

All three CAG biochemical constituents exhibited seasonal patterns of fluctuation. Protein content in April, June and July are not significantly different. April and June are significantly higher than all other months. Carbohydrate content is significantly higher in April than in all other months. June carbohydrate content is significantly lower than April and significantly higher than all other months. Lipid content was significantly higher in April than in all other months. Lipid content in May, June and July were not significantly different from each other but these three months were significantly lower than April and significantly higher than all other months.

Discussion

The major changes observed in both the component indexes and the content of the various biochemical constituents indicate that accumulation of organic reserve material is occurring on a seasonal basis in some body components of *Thais haemastoma.* We found the best component indicator of reproductive readiness in *T. haemastoma* to be the capsule–albumin gland complex. This has also been found true for *T. lamellosa* by Stickle (1973, 1975), *Fusitriton oregonensis* by Stickle and Mrozek (1973) and *Littorina irrorata* by Bistransin (1976). In both sexes copulation and capsule deposition, as indicated by decreases in component indexes, occur after April. The high values for component indexes in May and June suggest that spawning probably occurs intermittently during these months, is intensified after the July sampling data, and continues to occur at a reduced level throughout August. These results need to be substantiated by *in situ* observations of spawning members of the population.

The interpretation of this data is complicated by feeding patterns in *Thais haemastoma.* The secondary peaks observed in both the TDG and ODG may represent preparation for a second period of copulation and capsule deposition or reflect increased feeding and food availability. Peak spatsets of the oyster, *Crassostrea virginica,* occur in May–June and again in September (Pollard, 1973) thus altering food availability to the snails. Recent work by Spight and Emlen (1976) indicates that spawning is influenced greatly by food availability. In *T. emarginata,* for example, spawning rates were found to parallel barnacle densities and spawning frequency was adjusted to the food supply. It is also possible that increases in body component indexes observed in August and September represent storage of reserve material in preparation for the approaching winter months.

Seasonal changes in body component indexes have been observed in other species of *Thais.* Stickle (1973) found unimodal patterns of cycling in the TDG, ODG, and CAG of the west coast *T. lamellosa*, but found no seasonal patterns in the foot or RVM of either sex. The seasonal changes observed in the RVM of male specimens of *T. haemastoma* are difficult to explain. They may be due to cycles in the size of the prostate, the only male accessory organ. The RVM is not generally considered to be a major nutrient storage depot (Stickle, 1973). However, Bistransin (1976) found seasonal changes in the RVM of *Littorina irrorata.* This snail was also found to exhibit a bimodal reproductive pattern with peaks occurring in May and August.

The inverse relationship observed between percentage of body water and ambient salinity in males indicates that body water and total dry weight indexes are in part a reflection of changes occurring in the total body water in response to fluctuations in salinity. Body water in *Thais haemastoma* has previously been shown to vary between 72.9 and 81.9% during a simulated 30–10–30‰ S semidiurnal cycle, indicating that significant variations are occurring in response to short term fluctuations in ambient salinity (Stickle and Howey, 1975). However, it must be remembered that salinity determinations represent time–point recordings whereas in reality, salinity in the Barataria Bay system fluctuates considerably on both a daily and a seasonal basis (Hewatt, 1955). A temperature–dry weight index relationship was not observed in the male sample. This is probably due to the high index values recorded in the cold months of 1973 as compared with the low indexes recorded in the cold months of 1974. Since index data are not complete in 1973, the significance of the observation is unknown. The direct relationship between temperature and dry weight indexes in the female sample indicates that females mature reproductively at warmer temperatures and is in agreement with increases in the body component indexes observed during the warmer months of 1974.

The lack of a significant relationship between shell indexes and shell weight/shell length ratios indicates that changes in the indexes of the soft body components are due to actual changes in the weight relationship of the soft body parts. Seasonal changes in the shell indexes reflect changes in the sum total of the soft parts and body water and not a cyclical accumulation and mobilization of shell material. Stickle (1973) found similar results in the prosobranch, *Thais lamellosa.*

Changes in the biochemical content of *Thais haemastoma* were found to reflect changes in the respective body component indexes rather than fluctuations in constituent levels. This is not unexpected—most marine invertebrates do not possess distinct storage depots such as those found in the vertebrates (Giese, 1969; Stickle, 1975) and therefore any increases in biochemical substances must represent increases in cellular materials. Protein levels were somewhat higher than those recorded for marine prosobranchs where the Lowry (Lowry, Rosebrough, Farr and Randall, 1951) or Kjeldahl protein nitrogen determination methods were used. Foot protein in *T. haemastoma* comprised 60 to 74% of the entire biomass, while in *T. lamellosa* studied by Stickle (1975) and *Littorina irrorata* by Bistransin (1976) foot protein comprised only 30 to 40%. This may be a result of the differences in techniques used. The Lowry method has recently been shown to produce lower results than the classical biuret analysis when using BSA as a test standard (Bio–Rad Laboratory, Technical Bulletin 1015, 1977). Initial protein determinations on *T. haemastoma* using the Biuret and Lowry methods consistently gave lower values for the same tissue with the Lowry method.

Most of the biomass lost from the female visceral mass (comprised of ODG, CAG and RVM) in *Thais lamellosa* was deposited as capsule material (Stickle, 1973). The CAG produces all of the extraembryonic material surrounding the eggs, accounting for 39 to 45% of the visceral mass lost during aggregation in that species. The CAG in *T. haemastoma* is also the production site of extraembryonic material and the situation in this species is probably very similar to that observed in *T. lamellosa,* accounting for the large changes observed in protein, lipid and carbohydrate in the CAG. Stickle (unpublished data) found the egg capsule composition of *T. haemastoma* to vary with salinity. Capsules deposited at 30‰ S were composed of 53% protein, 7% lipid and 2% carbohydrate. In comparison, capsules deposited at 17.5‰ S were composed of 36% protein, 17% lipid and 2% carbohydrate. Capsule wall material is primarily protein with a small amount of mucoprotein (Fretter and Graham, 1962) while carbohydrate has been identified in the nutritive fluid of one species of pulmonate (Bayne, 1966). Lipid levels in the CAG are high when compared with the foot and RVM and may represent either a respirative energy source or a nutritive source for the developing embryos. Lipid has been found in the nutritive fluid of *Nucella* (=*Thais*) *lapillus* by Bayne (1968) and Stickle (1975) found lipid levels of 12.9% in the capsules of *T. lamellosa* produced at 30‰ S. Large changes in lipid content in some female components may be masking percent body water–salinity interactions, thus explaining the lack of a significant relationship between these two variables. In the black abalone, *Haliotis cracheroidii,* water levels decrease when lipid content in the ovary increases and water content falls considerably just prior to spawning (Giese, 1969).

The ovary and digestive gland are relatively unseparable and it is not clear whether accumulation of biochemical material is occurring in the ovary. It is possible that most increases in reserves occur in the digestive gland, with mobilization and nutrient transfer between the digestive gland and ovary prior to reproduction. Lawrence, Lawrence and Giese (1965) demonstrated an inverse relationship between the digestive gland and ovary in *Katharina tunicata,* suggesting transfer between these two organs. However, studies involving nutrient transfer are limited and much more remains to be done in this area. Nutrient storage in the gonad itself has been shown to occur in oysters and clams (Webber, 1970). Webber (1970) found no cyclical pattern in the digestive gland index of the abalone, *Haliotis cracheroidii,* and lipid levels in the gonad were found to correlate with the state of ripeness of eggs.

This research was partially supported by PRECOL Research Fellowship and was done as partial fulfillment of the requirements for the Master of Science Degree. A warm thank you is extended to Ms. Deborah French and Mr. David Randell for invaluable technical assistance and to Ms. Karen Westphal for help in preparing the figures. The advice given by Mr. J. Preston Newman on programming and running the statistical analyses is greatly appreciated. The authors wish to acknowledge with thanks the critical review of the manuscript offered by Dr. Thomas H. Dietz and Dr. J. P. Woodring.

Summary

1. Seasonal changes in the body component indexes of *Thais haemastoma* were studied over a 15 month period. Seasonal changes in the protein, carbohydrate and lipid level and content were determined for soft components over a 12 month period.

2. The capsule-albumin gland index was determined to be the best indicator of reproductive readiness in *Thais haemastoma.* This index cycled in a bimodal pattern, peaking in April and July.

3. Male testis–digestive gland and remaining visceral mass indexes cycled seasonally with major peaks in April and secondary peaks in September and

October, respectively. The female ovary–digestive gland cycled in a unimodal pattern, peaking in April. Changes in the shell index of both sexes were found to reflect changes in the sum total of the soft body indexes.

4. Seasonal changes in protein, carbohydrate and lipid content were found to be predominantly due to changes in component indexes and not to fluctuations in constituent levels.

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ARTEMIA HEMOGLOBINS: GENETIC VARIATION IN PARTHENOGENETIC AND ZYGOGENETIC POPULATIONS

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Brine shrimps are distributed throughout the world in inland salt lakes, coastal lagoons, and salterns in which salt is commercially produced by evaporation of sea water. The many populations have often been designated by one binomen, *Artemia salina* (Linnaeus) Leach. However, six groups of shrimp populations are reproductively isolated from one another (reviewed by Clark and Bowen, 1976). Four of these six sibling species, when reared in the laboratory under identical environmental conditions, can be identified by a combination of characterizations of esterases and NAD–dependent malate dehydrogenase isozymes (Bowen and Sterling, 1978).

When brine shrimps are reared in a hypersaline medium under a 100% oxygen atmosphere, no hemoglobin is detectable in the hemolymph. When shrimps are reared under air (20% oxygen), four hemoglobins appear: Hb–1, Hb–2, Hb–X, and Hb–3, in descending order of electrophoretic mobility on cellulose acetate (Bowen, Lebherz, Poon, Chow, and Grigliatti, 1969). Hb–X was seen so rarely that it was thought to be a genetic variant of one of the other three hemoglobins. It is now known that if shrimps are reared under a 7% oxygen atmosphere, all four hemoglobins are induced in almost every shrimp of the 30 populations studied in our laboratory. Hb–1 is an alpha homopolymer $(\alpha\alpha)_n$; Hb–2 is a heteropolymer $(\alpha\beta)_n$; Hb–X is a beta homopolymer $(\beta\beta)_n$. Because the beta locus has a higher threshold for induction than the alpha locus, Hb–X is present only when the oxygen partial pressure is greatly reduced (Bowen, Sterling, and Barkan, 1977). Hb–3 consists of 2n polypeptides which are neither alpha nor beta polypeptides (Sterling and Bowen, 1977). The value of n is estimated by means of two paradigms in the discussion section of this paper. The four proteins are coded by a minimum of three structural genes, none of which is closely linked to another (Sterling and Bowen, 1977).

The purpose of this paper is to characterize the prevalent hemolymph proteins in 30 populations which represent six sibling species of *Artemia*. Intrapopulation polymorphism in Hb–1, Hb–2, and Hb–3 will be contrasted in parthenogenetic and zygogenetic populations. The data suggest that five of the seven parthenogenetic populations are of monophyletic origin. The hemoglobins are used as markers to demonstrate that a rare male from a parthenogenetic population can transfer genes to a female from a zygogenetic population.

MATERIALS AND METHODS

The six sibling species are: *Artemia franciscana* Kellogg, 1906; *A. tunisiana* Bowen and Sterling, 1978; *A. urmiana* Gunther, 1900; *A. monica* Verrill, 1869;

A. persimilis Piccinelli and Prosdocimi, 1968; and *A. parthenogenetica* Bowen and Sterling, 1978. The locations of 27 populations within these six sibling species are listed in Table I. Detailed descriptions of most collection sites are given by Clark and Bowen (1976). San Francisco shrimps were collected as cysts from saltern A18 of the Leslie Salt Company at the southernmost tip of San Francisco Bay.

Mono Lake shrimps were collected as adults from the lake and maintained in lake water at 23° C for less than a week before electrophoresis. All other shrimps were hatched from cysts, fed on a mixed yeast diet, and maintained in glass vials (21 mm I.D.) in 5 ml of "medium D" at 23° C in the dark. The culture method has been described in detail (Clark and Bowen, 1976). *A. persimilis* and *A. franciscana* shrimps reached maturity (vitellogenesis in females) in two weeks. *A. tunisiana, A. urmiana,* and *A. parthenogenetica* required more time to mature (3–5 weeks). The matings were single-pair matings observed for a two week period. Shrimps were transferred from one vial to the next in glass pipets. These pipets were placed in boiling water for 5 minutes or more before they were employed to pick up shrimps of different genotype.

All shrimps were maintained under air (20% oxygen) until maturity. The hemoglobin patterns shown in Figures 1–3 and the data summarized in Tables I–III were from adults maintained under standard conditions (mixed yeast diet and air above culture medium D with no additional ferric salts) with three exceptions. Mono Lake shrimps were maintained in lake water. Some Tallaboa and Greater Inagua adult shrimps were maintained under an atmosphere of 10% oxygen and 90% nitrogen for two weeks to induce a higher level of hemoglobin because some shrimps in these two Caribbean populations had a higher threshold for induction of Hb–1 and Hb–2 (see pattern #4 in Figure 1).

For each electrophoretic analysis, the hemolymph from a single shrimp was collected and stored at 4° C for less than two hours prior to the run. The "Tris"–glycinate discontinuous buffer system, polyacrylamide slab gel (0.75 × 160 × 105 mm), water-cooled cell, stains, and procedures have been described (Sterling and Bowen, 1977). No stacking gel was used above the 6% acrylamide resolving gel. Two or more internal standards (samples containing Hb-1 with relative mobility of 100) were run on each gel. The standards were usually obtained from San Francisco and Kutch shrimps. Electrophoresis continued until the Hb–1 ^{100} had travelled 90 mm through the gel. Gels were stained with Coomassie Blue, a general protein stain. Relative hemoglobin concentrations were estimated on an EC180 recording densitometer. Mobility values were estimated from the trailing edge of Hb–1 and Hb–2 and from the center of Hb–3 and slow protein bands.

RESULTS

Hemoglobins: interpretation of electrophoretic patterns

Twenty-three zygogenetic populations were studied: the 20 listed in Table I (hemolymph studied by electrophoresis on slab gels) and three additional *A. franciscana* populations (studied only by electrophoresis on tube gels) from San Quintin, Baja California, Mexico; from Soap Lake, Washington, U.S.A.; and from James Island (Isla Santiago), in the Galapagos. In all of these 23

TABLE I

Relative mobility of the most frequent electromorph of each of three hemoglobins in 27 Artemia *populations (6% acrylamide slab gels). The prevalent electromorph in the San Francisco population was used as reference (with mobility of 100). Populations from adjacent localities are paired if the same pattern prevails in both. Each analysis was made on the hemolymph of one shrimp. Within each parthenogenetic population, all shrimps have both Hb-2 electromorphs. In the Hidalgo* persimilis *population, the faster and slower Hb-2 electromorphs are equally frequent and genotype frequencies follow the expected Hardy-Weinberg distribution.*

Population	Relative mobility of prevalent electromorph			Alpha locus polymorphism	Beta locus polymorphism	Number of analyses
	Hb-1	Hb-2	Hb-3			
Artemia franciscana						
1. Little Manitou Lake and Lake Chaplin, Saskatchewan, Canada	98	99	100	Yes	No	22
2. San Francisco Bay (SFR Pond) and Vallejo West Pond, Calif., U.S.A.	100	100	100	Yes	Yes	151
3. Moss Landing, Calif, U.S.A.	98	99	100	Yes	No	18
4. San Diego, Calif., U.S.A.	97	99	98	No	Yes	41
5. Great Salt Lake, Utah, U.S.A.	98	99	98	Yes	Yes	51
6. Quemado, New Mexico, U.S.A.	96	99	98	Yes	Yes	66
7. Kiatuthlanna Red and Green Ponds, New Mexico, U.S.A.	96	99	92	No	Yes	61
8. Pichilingue Island, Mexico	96	98	98	Yes	No	28
9. Tallaboa, Puerto Rico, West Indies	97	98	92	Yes	No	42
10. Greater Inagua Island, West Indies	97	98	98	Yes	No	9
11. Rockhampton, Queensland, Australia	98	99	100	Yes	No	15
Artemia tunisiana						
1. San Bartolomeo, Sardinia	97	97	87	No	No	10
2. Chott Ariana, Tunisia	97	97	87	Yes	No	19
Artemia urmiana						
1. Lake Urmia (Lake Rezaiyeh), Iran	100	104	98	Yes	Yes	17
Artemia monica						
1. Mono Lake, California	99	100	100	Yes	?	16
Artemia persimilis						
1. Carahue and Hidalgo, Argentina	96	97, 99	87	Yes	Yes	37
Artemia parthenogenetica						
1. Kutch and Madras, India	100	104	98	No	No	87
2. Odessa (Russia) and Sète (France)	100	103, 105	98	No	No	63
3. Yamaguchi, Japan	100	102, 106	98	No	No	26
4. Rottnest Island and Port Hedland, Australia	99	101	98	No	No	9

populations, there was a pronounced difference in male and female electrophoretic patterns. When shrimps were reared under air, the hemolymph of females contained more Hb–1 than Hb–2; the converse was true in males. Photographs which illustrate the sex differences in electrophoretic patterns have been published (Sterling and Bowen, 1977, pp. 427 and 435).

When shrimps were reared under "standard conditions" in culture medium D under air, the hemolymph contained an excess of alpha over beta polypeptides. Therefore, Hb–X, the beta homopolymer, was not detected. Hb–3 was always present in the hemolymph when shrimps were immature but often was not detected at 8 weeks of age or more. The amount of Hb–3 has been shown to decrease with age in a clone of shrimps of the same genotype, descended through five generations from a single parthenogenetic female (Bowen *et al.*, 1969). In the present study, Hb–3 mobilities showed slight variation in females of shrimps of the K3 clone descended through three generations from a single parthenogenetic Kutch female.

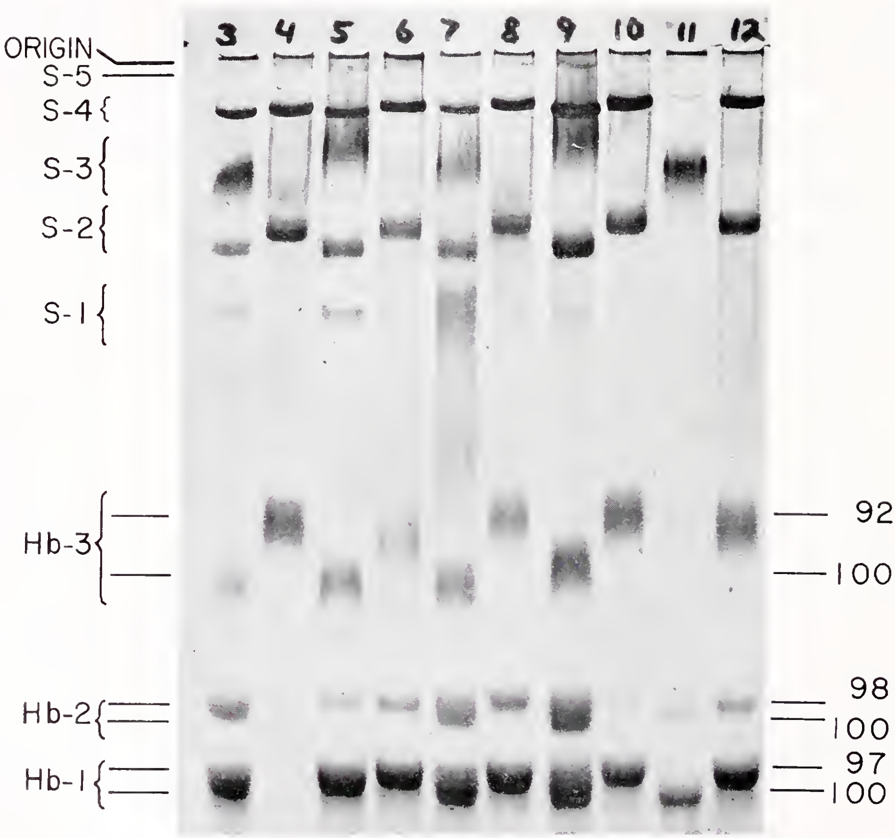

FIGURE 1. Hemolymph proteins from ten female shrimps (*Artemia franciscana*), showing three hemoglobins (Hb-1, Hb-2, and Hb-3) and five slow proteins (S-1 through S-5) which can be induced by low oxygen partial pressure in the 30 populations examined. Hb-X is not present because these shrimps were reared in culture medium under air. Hb-X appears in these populations when they are reared under an atmosphere of 7% oxygen. Shrimps #4, 6, 8, 10, and 12 are from Tallaboa; shrimps #3, 5, 7, 9, and 11 are from Vallejo. The common electromorphs in the Vallejo (and San Francisco) populations are assigned mobilities of 100. Shrimps #7 and #9 are heterozygous (α^{100}/α^{97}). Electrophoresis on 6% polyacrylamide slab gel, Tris-glycinate discontinuous buffer system, migration toward the anode (bottom of photograph).

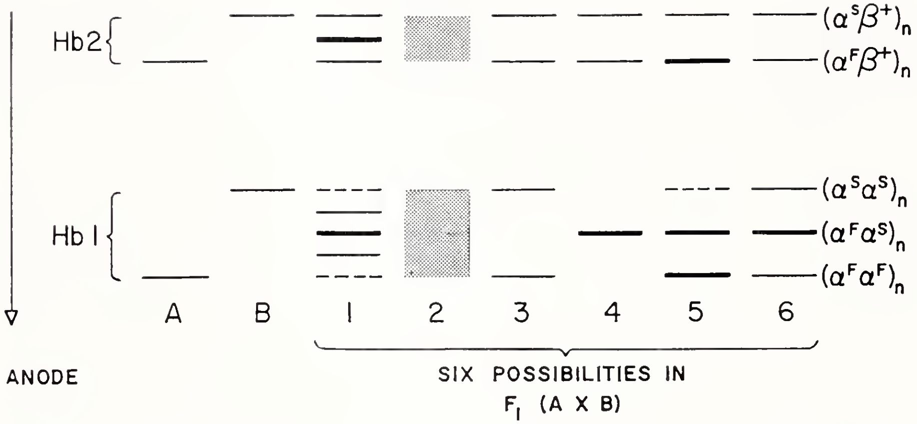

FIGURE 2. Predicted electrophoresis patterns of Hb-1 and Hb-2 in the hemolymph of F_1 progeny obtained by crossing homozygous shrimps A and B, genotypes $\alpha^F\alpha^F$; $\beta^+\beta^+$ and $\alpha^S\alpha^S$; $\beta^+\beta^+$, respectively. Possibility #6, a three-banded Hb-1 and two-banded Hb-2, was the most common pattern. It is the result of dissociation to dimers followed by reassociation of the dimers to form the native molecules. A few allelic combinations displayed pattern #5 (like #6 but with unequal amounts of the two alpha polypeptides) or #3 (further dissociation of $\alpha^F\alpha^S$ dimers to monomers before reassociation to form the native molecule). Possibility #2, a smear between the two parental extremes, was observed often in Hb-2 and more rarely in Hb-1. Patterns #1. (dissociation to tetramers) and #4 (greater affinity of unlike alpha polypeptides) were not documented in this study. An estimate of the value of n, the number of dimers in the native molecules, appears in the discussion section.

This suggests that this buffer system may not be optimum for this protein. Nonetheless, there are discrete mobility differences between populations (Figure 1 and Table I) and a three-banded Hb–3 is seen in the F_1 of certain racial crosses (Sterling and Bowen, 1977).

When two shrimps of different Hb–1 mobilities are mated, the F_1 progeny can be predicted to have six possible patterns (Figure 2). In most cases, pattern #6 was obtained: a three-banded Hb–1 and a two–banded Hb–2. The simplest interpretation is a rapid equilibrium of the native molecule with dimers. As the protein enters the gel, the dimers separate in the electric field, then reassociate to form native molecules composed of one kind of dimer (Bowen *et al.,* 1977). Possibilities #1 (dissociation to tetramers in Hb–1 and Hb–2) and #4 (preferential binding of unlike alpha polypeptides) were not documented in our study. Possibility #2 (a smear within the expected parental extremes of mobility) was often seen in Hb–2 but was only encountered in Hb–1 molecules containing the α^{99} and α^{98} alleles from San Francisco, Moss Landing, and Great Salt Lake. Possibility #3 (further dissociation of $\alpha^F\alpha^S$ dimers to monomers followed by reassociation to form the native molecules) was seen only in the combination of two San Francisco alleles (α^{100} and a rare α^{98}). Possibility #5 (like pattern #6 but with allelic products produced in unequal amounts) was seen when α^{92} from Quemado or α^{94} from Tallaboa was combined with α^{100} from San Francisco (or most other alleles). In both heterozygotes, the relative amounts of protein in the three bands of Hb–1 conformed to a binomial distribution, due to random assortment of the two alpha polypeptides. These statements about the possibilities in Figure 2 are made after study of these alleles in classical genetics pedigrees (Sterling and Bowen, 1977). Examples of the most common pattern (#6 in Figure 2) have been illustrated in photographs (Bowen *et al.*, 1977; Sterling and Bowen, 1977).

Hemoglobins: genetic variation

The relative mobilities of the most frequent Hb–1, Hb–2, and Hb–3 electromorphs in 27 populations are listed in Table I. The reference in each case is the prevalent electromorph in the San Francisco population (with mobility of 100). Because there is so much interpopulation variation within *Artemia franciscana,* no one hemoglobin mobility can be used to identify with certainty any one of the six sibling species. The most striking characteristic is the low value of the relative mobility of Hb–3 in *A. persimilis* and *A. tunisiana.* However, only two populations of each species were examined and this relationship may not hold in future studies. The fact that Hb–1 has the same mobility in two populations is not meant to imply that the alpha alleles are identical. The Hb–1 in the Yamaguchi population is more sensitive to heat denaturation for 30 minutes at 50° C than is Hb–1 in the San Francisco population, although both have relative mobilities of 100 (procedure in Sterling and Bowen, 1977).

In Table I, the presence of alpha locus polymorphism was usually seen as a three–banded Hb–1, patterns #5 and 6, and more rarely as the alternative patterns #2 and 3 in Figure 2. The presence of beta polymorphism was seen as a one-banded Hb–1 with two–banded Hb–2 (Figure 3, samples B and F) or a three-banded Hb–1 combined with a four-banded Hb–2 (sample E).

Allelic frequencies at the alpha locus in six populations are listed in Table II. Three of the populations of *A. franciscana* include shrimps at two adjacent localities where allele frequencies are similar. No evidence of significant deviation from the

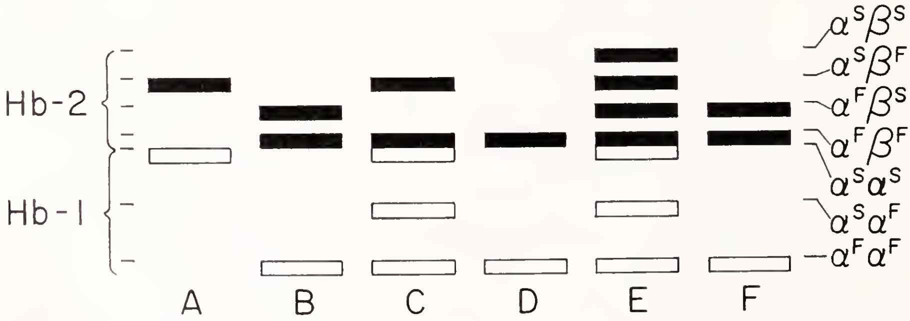

FIGURE 3. Tracing of photograph of electrophoretic patterns of six F_2 sibling shrimps from a parental cross of α^F/α^F; $\beta^F/\beta^F \times \alpha^S/\alpha^S$; β^S/β^S. Shrimps A and D are homozygous at both the alpha and beta loci and therefore each has only one species of Hb-1 and of Hb-2. Shrimps B, D, and F have the α^F/α^F genotype. Shrimps C and E have the α^F/α^S genotype and have a three-banded Hb-1. Shrimps A, C, and D are homozygous for the β^F allele. Shrimps B, E, and F are heterozygous at the beta locus. Shrimp E is heterozygous at both loci and the hemolymph pattern is a three-banded Hb-1 combined with a four-banded Hb-2. The genes are dervied from the San Francisco (SFR) and Quemado (QMD) populations. They are α_{SFR}^{100}, α_{QMD}^{92}, β_{QMD}^{F}, and β_{SFR}^{S}. Open bars: Hb-1; solid bars: Hb-2.

Table II

Hb-1 intrapopulation polymorphism in nine populations of A. franciscana *and in four populations of parthenogenetic shrimps. Data from three pairs of adjacent* franciscana *populations are pooled. Each analysis on hemolymph of one shrimp hatched from a cyst collected in the natural habitat.*

	Number of analyses	Frequencies of alpha alleles						
		100	99	98	97	96	92	Other
Artemia franciscana								
1. Lakes Chaplin and Little Manitou	22		0.03	0.97				
2. Kiatuthlanna ponds, New Mexico	61					1.00		
3. Quemado, New Mexico	66				0.02	0.87	0.11	
4. Pichilingue, Mexico	28				0.02	0.98		
5. SanDiego, California	41				1.00			
6. San Francisco and Vallejo	78	0.66						0.34*
Artemia parthenogenetica								
1. Kutch, Madras, Sète, Yamaguchi	176	1.00						

* San Francisco and Vallejo populations contained two or more alpha alleles which did not show the usual dissociation to dimers (pattern #6 in Figure 2).

Hardy–Weinberg distribution was observed in these shrimps hatched from cysts and reared in the laboratory. Heterozygosity is highest in San Francisco Bay shrimps. The exact value was not calculated because there are at least two alpha alleles which give patterns in heterozygotes which are difficult to interpret (dissociation patterns like those seen in Figure 2, possibilities #2 and #3). The other five *A. franciscana* populations cited in Table II are those which contain alpha and beta alleles which give unambiguous patterns (possibility #6 in Figure 2) and which have been studied in pedigrees (Sterling and Bowen, 1977). Hb–3 electromorph frequencies are not cited because resolution of this protein was not adequate in this buffer system. Hb–2 electromorph frequencies are not cited because this protein is a heteropolymer and both alpha and beta polypeptides show much variation in zygogenetic populations. Allelic frequencies at the beta locus are not given because these can be easily estimated only by determination of the relative mobilities of Hb–X, the beta homopolymer. Hb–X is consistently induced in every shrimp only when shrimps are reared for six weeks under 7% oxygen and this culture method was not used to maintain the shrimps described in Tables I through III.

Hemoglobins: genetic variation in threshold for induction

Both the alpha and beta loci are induced by low oxygen but the alpha locus has a lower threshold of induction. Thus, in San Francisco females reared by the standard method (under 20% oxygen), the amount of Hb–1 $(\alpha\alpha)_n$ exceeds the amount of Hb–2 $(\alpha\beta)_n$. When the same females are reared under 7% oxygen, the amount of Hb–2 exceeds that of Hb–1, and an additional protein, Hb–X or $(\beta\beta)_n$, appears in the hemolymph (Bowen *et al.*, 1977). In the present study, a lower threshold for induction (compared to the beta locus) was observed for the alpha locus in Kutch, Madras, Sète, Yamaguchi, Urmia, Kiatuthlanna, Quemado, San Francisco, and Great Salt Lake shrimps.

Parthenogenetic females (Kutch, Madras, Yamaguchi, and Sète) contained more hemoglobin than did non-parthenogenetic females which in turn produced more hemoglobin than males. The sex difference was apparent in the Caribbean zygogenetic populations (Tallaboa and Greater Inagua) where the total amount of Hb–1 and Hb–2 was low. Within the San Francisco and Tallaboa populations there was variation in amount of Hb–1 and Hb–2 induced when shrimps were reared under the same standard conditions. A Tallaboa shrimp with undetectable Hb–1 and Hb–2 is shown in Figure 1, sample 4. We succeeded in selecting inbred lines with greater amounts and lesser amounts of Hb–1 than in average San Francisco shrimps (stocks derived from San Francisco and Tallaboa, respectively).

Slower migrating hemolymph proteins: evidence that S–2 and S–4 contain globin

Five "slow proteins," which migrate more slowly than the hemoglobins, were sometimes present in hemolymph. They are designated S-1 through S-5, in descending order of relative mobility on 6% acrylamide gels (Figure 1). They are not present in hemolymph when adult shrimps are reared with a 100% oxygen atmosphere above the culture medium. When oxygen is reduced to 10% (with 90% nitrogen), at least a trace of each of the five slow proteins can be detected in some shrimps in each population.

When reared under standard conditions (defined in Materials and Methods section), the female shrimps reproduce by birth of free-swimming nauplii. When ferric EDTA is added to the medium (30 mg/liter), a high proportion of the females produce encysted blastulae (cysts). The addition of ferric salts increases the concentration of S-1 twenty-fold in females.

Within the K3 clone, descended in the laboratory from one Kutch parthenogenetic female, the outline of S-3 is smeared and its relative mobility is variable, suggesting that this buffer system is not appropriate for this protein. S-5 is very concentrated in the hemolymph of *A. persimilis*, but often undetectable in other populations. It is seen near the top of the gel in sample #9 in Figure 1.

Proteins S-2 and S-4 were readily detectable in *A. monica* and in all populations of *A. franciscana* and *A. persimilis*, where their relative mobilities were correlated with those of Hb-3. This correlation is evident in Figure 1 and also in inbred stocks with uniform mobilities of Hb-1 and Hb-2 but varying mobilities of Hb-3 (Sterling and Bowen, 1977, photograph on p. 427). When three-banded Hb–3 patterns were seen in F_1 and backcross progeny of racial crosses, the S-2 bands of the heterozygotes showed a smear between the parental extremes of S-2 mobilities. This correlation of mobilities of S-2, S-4, and Hb-3, which is even more prominent on 5% gels, indicates that these two slow proteins and Hb-3 contain a common polypeptide.

When shrimps from San Francisco, Quemado, Yamaguchi, Carahue, and Tunis were reared under standard conditions (defined in Materials and Methods section of this paper), the hemoglobins were red, peroxidase positive (indicating the presence of heme) and were not stained with Oil Red O (which indicates the absence of lipid). The slow proteins were colorless, peroxidase negative, and did not stain with Oil Red O. However, when shrimps from the same populations were reared under novel conditions (in a medium containing an additional 30 mg/liter of ferric EDTA, under 7% oxygen, and fed a diet containing algae), Hb-3, S-2, and S-3 were often stained by Oil Red O. When lipid was present, the proteins were often green with absorption maxima at 400 and 679 nm at pH 8.0. This suggests that carotene pigments were dissolved in the lipid. After cellulose acetate electrophoresis, the unstained bands of Hb-1, Hb-2, and Hb-3 were scanned for absorbance at 280 and 540 nm. The two values showed high correlation when shrimps were reared under standard conditions, suggesting a constant heme/protein ratio. The correlation was much lower when shrimps were fed a diet of algae or additional iron salts, suggesting variation in amount of heme bound to globin. The "Hb-3 band" was sometimes peroxidase negative and green before staining. This green protein was designated as "G-3" in an earlier study (Bowen *et al.,* 1969). When shrimps were reared under the same novel conditions, the five slow proteins were often both red before staining and peroxidase positive. Evidently, *Artemia* slow proteins and Hb-3 may be bound to varying amounts of heme and lipid *in vivo.*

Demonstration of interfertility of 13 Artemia franciscana *populations*

The evidence for reproductive isolation among the five zygogenetic sibling species listed in Table I has been reviewed by Clark and Bowen (1976). The evidence for interfertility of the populations grouped under *A. franciscana* will now be examined. In order to test for reproductive isolation, single pair matings were made between each unknown population and either San Francisco (SFR) shrimps or shrimps "cross fertile with San Francisco" (CRSFR). The CRSFR stock carries the recessive gene, ω, for white eyes and has been repeatedly backcrossed to SFR shrimps. The gene for white eyes is partially sex-linked; *i.e.,* it is on the homologous portion of the sex chromosomes (Bowen, 1965). This genetic marker was utilized in the racial crosses to exclude the possibility of gynogenesis (pseudogamy).

The data from cross-fertility tests of 12 populations are shown in Table III. In each cross and reciprocal cross, fertile F_1 and viable F_2 progeny were seen. When the white-eye gene was present, the expected F_2 segregation ratio (75% wild, 25% white) was seen. Omitted from the table are the control values for fertility in intrapopulation crosses. These ranged from a low of 6/29 (21%) in Quemado to 19/21 (90%) in Pichilingue shrimps. Because the San Francisco population is not reproductively isolated from the 12 populations in Table III, it is concluded that all are populations of *Artemia franciscana.*

Progeny of exceptional males from parthenogenetic populations

In the progeny of parthenogenetic females reared in our laboratory, we discovered four males. Two were found among the 1000 Madras females examined, one among 166 Sète females, and one among approximately 500 Yamaguchi females. Each male was mated to females from zygogenetic species. Three males (one each from Madras, Sète, and Yamaguchi) sired progeny which consisted of males and non-parthenogenetic females in approximately equal numbers. In the two crosses detailed below, the possibility of pseudogamy (gynogenesis) is excluded by the transfer of genetic markers from males to their progeny.

TABLE III

Hybridization of shrimps from 12 populations with shrimps from San Francisco (SFR) or an inbred line of white-eyed shrimps (w/w *genotype*) *which are "cross-fertile with San Francisco" (CRSFR). In the parental cross, each wild-type shrimp hatched from a cyst collected in the natural habitat.*

Parental cross Female × Male	Fertile pairs / total matings	Normal F_2 progeny obtained from each fertile parental pair	Normal F_2 segregation of gene (*w*) for white eyes
Great Salt Lake × CRSFR	19/34	Yes	Yes
CRSFR × Great Salt Lake, Utah	14/22	Yes	Yes
Greater Inagua × SFR	1/1	Yes	—
Greater Inagua × CRSFR	3/4	Yes	Yes
SFR × Greater Inagua Island	5/11	Yes	—
Kiatuthlanna Green × CRSFR	1/1	Yes	Yes
CRSFR × Kiatuthlanna Green Pond	4/6	Yes	Yes
Kiatuthlanna Red Pond × CRSFR	12/15	Yes	Yes
CRSFR × Kiatuthlanna Red Pond	4/4	Yes	Yes
Little Manitou × CRSFR	3/5	Yes	Yes
CRSFR × Little Manitou, Saskatchewan	8/10	Yes	Yes
Moss Landing × CRSRF	7/11	Yes	Yes
CRSFR × Moss Landing, California	9/10	Yes	Yes
Pichilingue × CRSFR	27/58	Yes	Yes
CRSFR × Pichilingue, Mexico	9/25	Yes	Yes
Quemado × CRSFR	6/11	Yes	Yes
CRSFR × Quemado	11/22	Yes	Yes
SFR × Quemado, New Mexico, U.S.A.	12/17	Yes	—
Rockhampton × SFR	3/8	Yes	—
Rockhampton × CRSFR	6/6	Yes	Yes
SFR × Rockhampton, Australia	2/2	Yes	—
San Diego × CRSFR	7/9	Yes	Yes
CRSFR × San Diego, California	9/13	Yes	Yes
San Quintin × CRSFR	3/4	Yes	Yes
CRSFR × San Quintin	2/2	Yes	Yes
SFR × San Qunitin, Mexico	9/13	Yes	—
Tallaboa × CRSFR	4/5	Yes	Yes
CRSFR × Tallaboa	2/2	Yes	Yes
SFR × Tallaboa, Puerto Rico	12/20	Yes	—

The Madras male (found by G.S.) was mated to the white-eyed CRSFR stock (*A. franciscana*) and sired 37 male and 38 female F_1 progeny, all of which had black eyes. Eleven F_1 females were isolated and none produced progeny over a five-week period. Another 17 were mated to their brothers and produced an F_2 generation with about equal numbers of males and females and the expected segregation data for the white and sex loci. Forty-three of the F_2 females were isolated for a period of four weeks and none was parthenogenetic. We conclude that the gene (or genes) for parthenogenetic reproduction cannot be transmitted through the male.

The Yamaguchi male (found by S.T.B.) was mated to an *A. urmiana* female and sired two F_1 progeny. The F_1 male was mated to an *urmiana* female and produced seven F_2 offspring. The three F_2 females were not parthenogenetic. The exceptional Yamaguchi male and his F_1 daughter were tested for hemoglobins and two esterase isozymes. The hemolymph of the Yamaguchi male showed the hemoglobin pattern characteristic of Yamaguchi (the unique two bands of Hb-2 with mobilities of 1.02 and 1.06) but with greater amounts of Hb-2, relative to the amount of Hb-1, than is found in female Yamaguchi shrimps. His F_1 daughter had these two Hb-2 bands but the area between them was smeared, perhaps due to hybridization with the Hb-2 (with relative mobility of 104) typical of *urmiana*. The entire bodies of the exceptional male and his daughter were macerated and tested for the two "fast esterases" (Bowen and Sterling, 1978). The Est-2 was a single band of mobility intermediate between that of *A. urmiana* and the Yamaguchi population. The mobility of Est-1 could not be stated with certainty.

Although the transfer of genes from parthenogenetic populations to zygogenetic populations has been documented, this phenomenon must be extremely rare in the natural habitat due to the low incidence of male offspring of parthenogenetic females. Stefani (1964) reared Cagliari diploid parthenogenetic *Artemia* in the laboratory and reported the incidence of male offspring to be 0.004.

Discussion

The taxonomy of *Artemia* is in a state of flux because the morphological differences between sibling species vary with age, sex, and environmental influences. Many populations have been reported to consist of mixtures of sibling species (reviewed by Halfer-Cervini, Piccinelli, Prosdocimi, and Baratelli-Zambruni, 1968; Barigozzi, 1974). Early taxonomists assigned species names to shrimps at each locality and these names were often based on preserved specimens of different ages which were collected from salterns of different salinities. Therefore, we are aware that a few of the species names cited in this paper may be supplanted because some species might contain populations with name priority which have not yet been characterized by isozyme analysis nor by studies of reproductive isolation. Furthermore, taxonomic convention dictates that parthenogenetic clones bear the species name of the biparental population from which they are derived (Mayr, 1969). If the parthenogenetic clones can be shown (by inference from isozyme analysis) to be descended from, for example, *A urmiana*, then the name *A. parthenogenetica* would be supplanted by the more conventional *A. urmiana parthenogenetica*.

Taxonomic convention requires that the name *A. salina* be assigned to the shrimps observed in 1755 at the type locality (Lymington, England). Kuenen and Baas-Becking (1938) reviewed the contributions of Linnaeus, Lamarck, and Leach to the taxonomy of *Artemia* and printed a translation of the original description (by Schlosser in 1755) of the zygogenetic population at Lymington which was named by Linnaeus in 1758. Because two zygogenetic sibling species have been reported at one site in Europe (Halfer-Cervini *et al.*, 1968), it is uncertain which, if any, of the species in the present study is identical to *A. salina Linnaeus.* It will be confusing to use the binomen *A. salina* to designate one of the European sibling species. Most biochemical studies have utilized California brine shrimps (*A. franciscana*) but have erroneously designated them as *A. salina* because the presence of sibling species was not recognized. We suggest that *A. salina* be discontinued as a name for populations in existence today.

The "slow proteins," which migrate more slowly than the hemoglobins, are a prominent feature in electrophoretic patterns of *Artemia* hemolymph proteins on 6% polyacrylamide gels (Figure 1). A discussion of these proteins is appropriate because two of the slow proteins, S-2 and S-4, contain a polypeptide in common with Hb-3. In starch gel electrophoresis of the hemolymph of *Parartemia zietziana,* Manwell (1978) found a colorless protein which migrated more slowly than the hemoglobins and which could bind heme *in vitro.* Perhaps the relationships of the four hemoglobins and five slow proteins of *Artemia* will someday be elucidated and they will be numbered consecutively. However, a new nomenclature would be premature at this time.

In order to estimate the value of n, the number of dimers in Hb-2 $(\alpha\beta)_n$, one must understand the structure of both the slow proteins and the hemoglobins. There is substantial agreement on the molecular weights of the native hemoglobin molecules. Hb-1, Hb-2, and Hb-3 have the same sedimentation coefficient (11.3S), which suggests a molecular weight of about 230,000 to 260,000 (Waring, Poon, and Bowen, 1970; Bowen, Moise, Waring, and Poon, 1976; Moens and Kondo, 1976, 1978; Manwell, 1978). The four hemoglobins and the genetic variants discovered to date have the same molecular size and conformation. Evidence for this is the fact that the relative distance between the proteins remains constant on cellulose acetate (no molecular sieving) as well as on 4, 5, 6, and 7.5% acrylamide gels (Bowen *et al.,* 1977). The molecular weight of the smallest hemoglobin subunit has been estimated from iron content and by relative electrophoretic mobility in SDS gels. Estimates of iron content of 0.29 and 0.299% (Bowen *et al.,* 1976; Moens and Kondo, 1978; respectively) have suggested a molecular weight per heme of 15,000–19,000 daltons. However, widely disparate conclusions were drawn from the SDS gel experiments. Moens and Kondo (1978) estimated that the smallest subunits obtained in SDS gels were of unequal size, 50,000 and 80,000 daltons, suggesting that each polypeptide was associated with more than one heme. They concluded that the native molecule was a dimer and that each of the two subunits (126,000 daltons) was composed of seven similar repeat units covalently linked to one another. Bowen *et al.* (1976) concluded that each polypeptide was the same size, about 15,000–19,000 daltons, and was associated with only one heme. The native molecule would consist of 12 polypeptides. It is now known that much free globin (hemoglobin apoprotein) is present at times in the hemolymph, suggesting that the previous estimate of 0.29% iron content in hemoglobins of saltern shrimps might have yielded too high an estimate of subunit molecular weight. The highest value for iron content of *Artemia* hemoglobin to be reported is 0.42% for one sample of Hb-1 (Moise and Bowen, unpublished). Because the number of polypeptides in the native molecule is a matter of controversy, the value of n may be unity or 6 or more, according to the paradigms of Moens and Kondo (1978) and of Bowen *et al.* (1977), respectively.

Because the ionic composition of the waters inhabited by *Artemia* varies more than that of any other aquatic metazoan, correlations were sought of allele frequencies with characteristics of habitat. In this study, shrimps originated from waters where the average salinity ranged from 54 to 210 g/liter (Mono Lake and Quemado, respectively). The ratio of chloride to carbonate ranged from 1.1 (Kiatuthlanna Green Pond and Mono Lake) to 810 (Lake Urmia). The ratio of sodium to potassium ranged from 8 (San Francisco) to 173 (Quemado). The ratio of chloride to sulfate ranged from 0.5 (Little Manitou) to 90 (Kiatuthlanna Red Pond). An interesting contrast was two inland lakes (Quemado and Kiatuthlanna Red Pond) only 85 km apart with chloride/carbonate ratios of 700 and 3 and chloride/sulfate ratios of 18 and 90, respectively. In these lakes, the most frequent electromorphs of Hb-1 and Hb-2 had identical mobilities. Although the prevailing Hb-3 was not the same in both populations, the most frequent Hb-3 mobility in one was the rare Hb-3 mobility in the other. Distinctive characteristics of electrophoretic pattern could not be associated with high total salinity, nor with high potassium, carbonate, or sulfate composition. It is possible that the electromorphs seen in different populations are the result of chance fixation of neutral alleles. The chemical composition of the waters inhabited by *Artemia* has been reviewed by Cole and Brown (1967).

A search for geographical patterns and latitudinal clines of alleles also yielded negative results. Although 2000 km apart, two parthenogenetic populations, Madras and Kutch, had identical mobilities for the three hemoglobins. Another two populations, San Francisco and Moss Landing, had different mobilities of the prevalent alleles although they were only 75 km apart.

Asexual reproduction is not necessarily associated with genetic uniformity. Saura, Lokki, Lankinen, and Suomalainen (1976) observed 20 isozyme loci in 127 populations of parthenogenetic tetraploid weevils of monophyletic lineage. The majority of loci were polymorphic. Genetic variation was evident within and between weevil populations.

In the present study, there was no intrapopulation variation in Hb-1 or Hb-2 phenotype among the parthenogenetic shrimps. This suggests that populations such as Kutch or Sète (57 shrimps from each population examined) are each derived from one clone which has recently invaded the habitat. It is not possible to comment on polymorphism in Hb-3 because there were slight variations in Hb-3 mobility within the progeny of a single Kutch female. The buffer system may be inappropriate for adequate resolution of this protein. The data in Table I suggest that Kutch and Madras are recently derived from the same clone, as are the Sète-Odessa pair and the Port Hedland-Rottnest pair. In Table I, if two Hb-2 mobilities are indicated in a parthenogenetic population (*e.g.*, Yamaguchi), every shrimp in that population had both bands of Hb-2. The Yamaguchi shrimps may all be heterozygous (B_1^{102}/β_1^{106}) or may show expression of a duplicate locus (homozyous genotype $\beta_1^{102}/\beta_1^{102}$; $\beta_2^{106}/\beta_2^{106}$).

Five of the seven parthenogenetic populations show striking similarities. Kutch, Madras, Sète, Odessa, and Yamaguchi have identical mobilities of Hb-1. All have Hb-2 species with relative mobilities greater than 100. They have identical patterns of esterases and NAD-dependent MDH isozymes which set them apart from the Port Hedland parthenogenetic population and ten zygogenetic populations representing *A. tunisiana, A. urmiana,* and *A. franciscana* (Bowen and Sterling, 1978). This suggests that either these five parthenogenetic populations, all of which live in coastal salterns, have had the same alleles selected due to similarity of environment or all have descended with mutation from a monophyletic origin. The first explanation seems unlikely because zygogenetic *A. franciscana* shrimps from coastal salterns (San Francisco, Tallaboa, Moss Landing, Pichilingue, for example) have evolved different characteristic patterns of hemoglobin mobilities. The second explanation is more plausible: the five have originated from a single "transformation to parthenogenetic reproduction event" from a zygogenetic population. It will be interesting to see if this generalization holds as these strains are characterized further for biochemical traits.

This work was supported by grant HL-11455 from the National Institutes of Health.

SUMMARY

1. Twelve populations of *Artemia* were found to be crossfertile with San Francisco shrimps, which indicates that they are *Artemia franciscana.*

2. Three hemoglobins (Hb-1, Hb-2, and Hb-3) were induced in shrimps from 30 populations. Although many populations had unique patterns (considering relative mobilities of all three hemoglobins) there was so much interpopulation variation among 14 populations of *A. franciscana* that no one protein was diagnostic of any one of the five sibling species of zygogenetic shrimps.

3. Intrapopulation polymorphism of Hb-1 and/or Hb-2 was seen in most zygogenetic populations but was not detected in parthenogenetic populations. This suggests that parthenogenetic populations such as Kutch or Sète (57 shrimps examined from each) are each descended from a single clone which has recently invaded the habitat. Five of the seven parthenogenetic populations have Hb-1 and Hb-3 electromorphs with the same relative mobilities, suggesting that they may have a monophyletic origin.

4. Two exceptional males (progeny of parthenogenetic females reared in the laboratory) were mated to females from zygogenetic populations. The progeny were equal numbers of males and non-parthenogenetic females. Transfer of genes from one exceptional male to an *A. urmiana* female was documented with three genetic markers.

5. The relationship of the hemoglobins and the "slow proteins" of the hemolymph is discussed.

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HABITAT ADAPTATIONS OF NORTH AMERICAN CAPRELLID AMPHIPODA (CRUSTACEA)

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Caprellid amphipods are common marine peracarid crustaceans that usually occur epibiotically and typically move on their substratum by crawling. Crawling consists of grasping the substratum with the gnathopods, releasing the grasp of the pereopods, and pulling the pereopods to a point just posterior of the gnathopods. At this point, the body is in an Omega-like configuration. The gnathopods are then released, the body is straightened, and the gnathopods again grasp the substratum. Caprellids do possess limited swimming capabilities, but powerful or directional swimming over a distance in excess of 15 cm has not been reported.

Specific substratum preferences are reported for a few caprellids, but the majority of species occur on a variety of organisms. The mechanism whereby a caprellid selects a substratum is unknown. As caprellids have direct development and, perhaps, limited powers of dispersal, Mayer (1882), McCain (1968), and Caine (in prep.) have indicated that rafting is the most frequent dispersal mechanism.

Some species with specific habitats have morphological adaptations to their substrata. Even those species occurring on different substrata may exhibit some substratum-specific morphological adaptations (McCain, 1968). Collections from Washington (Puget Sound, U.S.A.) yielded *Caprella gracilior* Mayer on the starfish *Luidia foliolata* Grube, *Mayerella banksia* Laubitz free on the bottom (not epibiotic), and *Perotripus brevis* (La Follette) on tubes of *Phyllochaetopterus prolifica* Potts. Each species exhibited morphological variations from "normal" caprellids.

This paper reports on the substratum-related morphological variations of North American caprellids, with emphasis on those appendages used in crawling.

MATERIALS AND METHODS

The caprellids are divided into four habitat categories: cosmopolitan substrata, starfish epibionts, gorgonian and bryozoan epibionts, and free on the bottom. Steriomicroscope observations on locomotion and stationary posturing were conducted on 15 species: *Caprella laeviuscula* Mayer (occurring in the northeastern Pacific, hereafter indicated by P), *C. natalensis* Mayer (P), *Caprella striata* Mayer (P), *Deutella californica* Mayer (P), *Luconacia incerta* Mayer (occurring in the northern Gulf of Mexico, hereafter indicated by GM), *Metacaprella kennerlyi* (Stimpson) (P), and *Tritella pilimana* Mayer (P)—cosmopolitan substrata; *Caprella greenleyi* McCain (P) and *C. gracilior* Mayer (P)—starfish epibionts;

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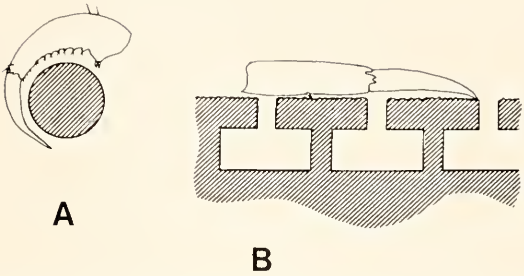

FIGURE 1. Grasping of the substratum by caprellid pereopods: A, encirclement (= typical mechanism); and B, grasping flat surface (= starfish epibionts).

Caprella penantis Leach (GM), *Paracaprella pusilla* Mayer (GM), and *P. tenuis* Mayer (GM)—bryozoan and gorgonian epibionts; and *Mayerella banksia* Laubitz (P) and *Perotripus brevis* (La Follette) (P)—free on the bottom.

Feeding mechanisms were observed following the methods of Caine (1977) with the specimens on their natural substrata. Stomach contents were examined in the segment of the digestive tract from the mouth to the end of pereonite III. More details are included for those species which only have been described taxonomically.

Species identifications were made with the aid of monographs by McCain (1968) and Laubitz (1970) and a paper by McCain (1969).

OBSERVATIONS AND RESULTS

Cosmopolitan substrata

All of the species occur on algae and hydroids, however, in my collections, *Caprella natalensis* occurs most frequently on Rhodophyta, *Deutella californica* on hydroids, and *Luconacia incerta* on attached *Sargassum*. All of the substrata are highly branched; the circumference of most branches of the substrata are small enough to allow encirclement by the pereopods so that the palmar portion of the propod contacts the substratum (Fig. 1A). Grasping spines, which occur proximally on the propodus (Fig. 2A), are in contact with the substratum.

An upright stance may occur when an animal is stationary. Grasping the substratum with the pereopods, pereonites VI and VII are in contact with the substratum. An angle of 90° is made at the articulation of pereonites V and VI; the remainder of the body is held vertically erect (Fig. 3A). Pereopod 5 of all of the cosmopolitan species examined herein is 6-segmented and usually inserted posteriorly on pereonite V.

Feeding mechanisms are dependent upon antennal and mouthpart setation; those species with swimming setae on the antennae are scrapers/filter-feeders, whereas those species without swimming setae obtain food by predation and scavenging (Caine, 1977).

Starfish epibionts

Caprella gracilior is a large caprellid occurring in dense populations on the aboral surface of *Luidia foliolata* Grube. *Luidia* is a large, subtidal starfish with

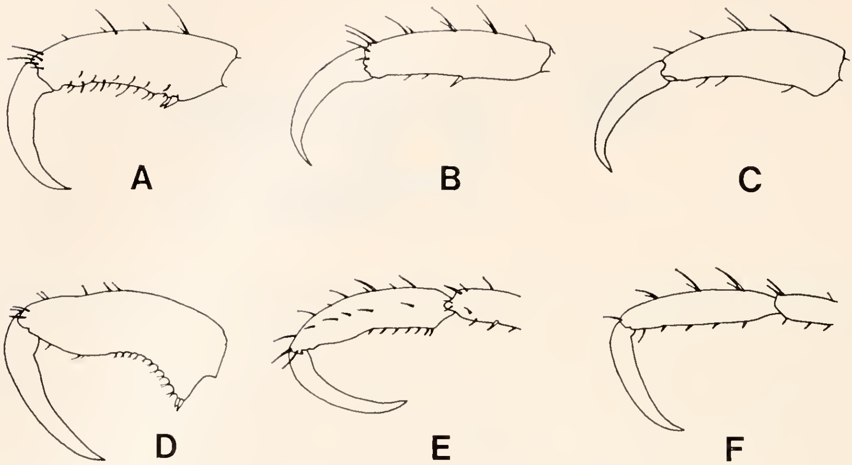

FIGURE 2. Structure and spination of the propod and dactyl of pereopod 7 as related to substratum type: A, cosmopolitan substrata (*Caprella laeviuscula*); B, starfish epibiont (*C. gracilior*); C, gorgonian epibiont (*C. penantis*); D, bryozoan epibiont (*Paracaprella pusilla*); and E and F, free on the bottom (*Mayerella banksia* and *Perotripus brevis*, respectively).

close-set paxillae and lacking prominent pedicellaria. The aboral surface is covered with small spines arising from the paxillae. Space between the paxillae varies slightly as the starfish moves its arms. General morphology of *C. gracilior* includes lack of prominent spination, sparse setation on the body and pereopods, the slender pereopods 5–7, and no true palmar development with a median grasping spine on the cylindrical propodi (Fig. 2B).

Crawling is typical (Fig. 1B) except that the substratum (starfish) is not encircled by the pereopods. Predominant feeding mechanisms are scraping and filter-feeding, but predation occurs frequently. Filter-feeding is similar to other caprellids (Wetzel, 1932; Caine, 1974, 1976, 1977); swimming setae on the antennae passively filter settling material from the surrounding water.

As observed, scraping is restricted to the capture of mucous secretions of the starfish and the settled material contained therein. Mucous capture occurs while the caprellid is stationary, but mucous strands are frequently located while the caprellid is crawling. Mucous strands are picked from the starfish with the first gnathopods, transferred to the maxillipeds and inner mouthparts, and ingested.

If a small organism (<3 mm) swims between the laterally spread gnathopods II and the anteriorly directed antennae while *C. gracilior* is in an upright stance, capture occurs as both pairs of antenna flex ventrally concurrent with medial movement of gnathopods II. During medial movement, the gnathopods are rotated so that the grasping margins contact the prey as the prey is forced into the grasp of gnathopods I. Epibenthic prey are captured as *C. gracilior* extends laterally from the arms of the starfish. The antenna do not assist in prey entrapment with this mode of predation, but the antennules did appear to guide prey into the grasp of gnathopod I by causing the potential prey to alter its direction of movement. When the prey moves near the cephalon, it is grasped by the first gnathopods. Occasionally the mesioventral (broadside) portion of the propod of gnathopods II aid in prey capture by forcing the prey into the grasp of the first gnathopods.

This caprellid cleaned its antennae frequently. The dactyli of the maxillipeds and of the first gnathopods are crossed with the antennae that is being cleaned in the center. As the antenna is drawn anteriorly, material is transferred from the antenna to the maxillipeds. The material may be passed orally and ingested, or it may be concentrated on the dactyl of the maxillipeds and discarded. The fate of cleaned material is dependent on the amount of material on the antennae. The more material that is present, the greater is the chance that it will be discarded.

Caprella greenleyi was captured from intertidal algae and the aboral surface of *Henricia leviuscula* (Stimpson) inhabiting tidepools. *Henricia* is a small starfish that lacks a pedicellaria. *C. greenleyi* has small spines on pereonites V–VII, robust pereopods 5–7 with slight palm development, and each pereopod has a pair of grasping spines on the propod. Setation is short, but numerous, on the pereopods.

Feeding modes are restricted to scraping and filter feeding, with the major portion of the diet obtained by scraping. Crawling, antennal cleaning, and feeding are as described for *C. gracilior.*

Bryozoan and gorgonian epibionts

Adaptations to gorgonian hosts have been described by McCain (1968) for *Caprella penantis* and *C. equilibra.* He found a reduction in the ventral spine between gnathopods in *C. equilibra. C. penantis* has a reduction in pereopod setation and a reduction in the size and number of grasping spines (Fig. 2C). Caine (1974) examined feeding in *C. penantis* and observed the mucous secretions from the gorgonian host were used to increase particle entrapment by the antennae. However, most food materials are obtained by scraping the encrusting diatoms and detritus laden mucus from branches of the gorgonian.

Gorgonian branches are small enough to be partially encircled by the pereopod, and crawling is typical for the suborder.

Paracaprella pusilla and *P. tenuis* are most frequently captured among erect branching byrozoans (*e.g., Amathia* sp. and *Bugula neritina*). Both caprellid species have concave propodi on the pereopods which are proximally adorned with a pair of grasping spines and several knobs which increase in size proximally (Fig. 2D). The knobs have several short apical setae. Both species crawl in the typical manner, but when assuming an upright stance, *P. tenuis* frequently uses pereopod 5 to grasp a different substratum branch than that which is grasped by pereopods 6 and 7. Ambush predation (Caine, 1974, 1977) was the most frequently observed method of obtaining food for both species, but both commonly utilized alternate feeding modes.

Free on the bottom

Mayeralla banksia is a small caprellid existing free in dense populations on muddy sand substrata. Densities up to 38,750 m^{-2} were estimated from the largest sample (31 caprellids in an 8 cm^2 sample). *M. banksia* is seasonally abundant with populations most dense in the summer months, but ovigorous females have been

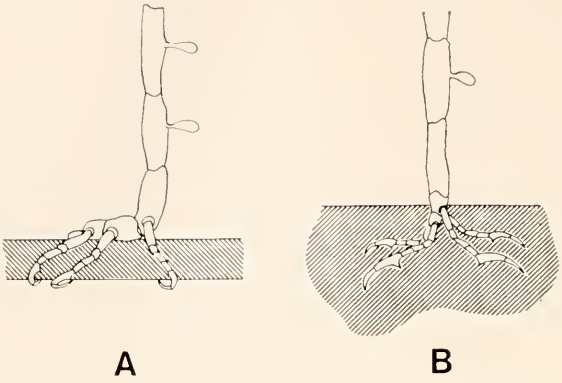

FIGURE 3. Upright stance of caprellids indicating the vertical height increase with the modified stance: A, typical stance with six-segmented pereopod 5; and B, modified stance with reduced pereopod 5.

captured in July and August. Morphological characters include the lack of swimming setae, overall body smoothness, pereopod 5 inserted near the midlength of pereonite V and reduced to three segments, and pereonite VII reduced. The propod of the pereopods has a weakly developed palm. Spines occur on the proximal half of the propod and spine density increases proximally; the dactly is smooth, without spines or serrations (Fig. 2E). The anterior margin of the pereopods, the side facing the substratum when assuming an upright stance, is lined with spines from the merus to the propodus.

When stationary, *M. banksia* assumes an upright stance with the entire body held vertically erect (Fig. 3B). Support is achieved by placing the appendages on the surface of the substrata rather than by grasping; articulations of the short ischium allow an angle of 90° to be formed with the basis and the joints distal to the ischium-merus flexure remain outstretched. During crawling, the substrate is grasped by projecting the dactyl of the second gnathopods downward, into the substratum. Otherwise, crawling is similar to that of other caprellids except that the posterior portion of the body is anchored by the dactyls of pereopods 6 and 7 only.

Mayerella banksia readily releases its grasp of the substratum when physically disturbed and swims. Swimming movements are typical of caprellids (described by Jessen, 1969 and others), and the caprellid settles to the bottom after covering a distance of several cm (seldom more than 8–10 cm in still water).

Although the substratum appears to be highly organic (carbon examinations were not conducted), *M. banksia* relied on predation to obtain its nutrients rather than utilize the substratum directly. Prey organisms are mainly harpacticoid copepods, which are abundant in or on the substratum, but some small ostracods and gammarid amphipods are also captured. Scavenging occurs less frequently, but *M. banksia* does pick small objects from the substratum and ingests adhering material.

Mayerella banksia frequently cleans itself, especially the anterior appendages. Some of the material is ingested, but usually it is discarded. Antennal cleaning is similar to that of other caprellids with mandibular palps (Caine, 1976), *i.e.,* the dactyli of the maxillipeds are uncrossed with the ventral area of the antenna contacted by the mandibular palps.

Perotripus brevis is a small caprellid occurring as epifauna on tubes of *Phyllochaetopterus prolifica* Potts in the very low intertidal (−0.9 m, below MLW). Only three collections of *P. brevis* were made, and these occurred in June and July. Ovigerous females and juveniles were found in the collections on all three occasions. This species lacks swimming setae, pereopod 5 is inserted near the midlength of pereonite V and is reduced to three segments, and pereonites II and III are pear-shaped. The pereopods are similar in structure to those of *Mayerella banksia,* except there is no palm development on the propod, spination occurs on the entire margin of the propod, and there is no proximal spine concentration on the propod (Fig. 2F).

The upright stance of *Perotripus brevis* is similar to that of *Mayerella banksia* (Fig. 3B), except that the sand grains cemented together to form the worm tubes are grasped by the appendages in the typical manner (Fig. 1A). The sand grains are grasped; encirclement of the worm tube does not occur. Swimming occurs only when repeatedly stimulated. The initial escape response is to crawl among the mass of worm tubes and then to remain still.

Perotripus brevis feeds primarily be predation. Harpacticoid copepods (0.20–0.31 mm) are the major prey. Prey are captured by rapidly bending the body and grasping the prey by the first gnathopods. The gut contents of *P. brevis* appeared to contain a lot of detritus and diatoms, but this material may have been the stomach contents of the harpacticoid prey. Abundant protozoans on the worm tubes may have also been ingested and rapid digestion of them would leave no trace in the stomach contents.

Observations were also made on intraspecific aggression of *Perotripus brevis.* Aggressive encounters resembled pushing matches where two opponents met, aligned their pereonites II and III, and pushed. The victor was the individual that pushed the opponent's pereonite V past vertical. The subordinate caprellid crawled away while the dominant remained stationary.

Discussion

Podocerid amphipods, thought to be morphologically similar to the ancestral caprellid, include a group of species that occur epibiotically (Enequist, 1949; McCloskey, 1970). The pereopods (5–7) have numerous spines on the carpus or proximally on the propodus, and the palm is weakly developed (Laubitz, 1977). These forms have generalized grasping articles that, through centralization of the grasping spines, the inclusion of a palm, and increased prehensility (= rotation and subchelated) gave rise to the generalized caprellid pereopod structure (Fig. 2A). The primitive caprellids, *Caprogammarus* and *Cercops,* cosmopolitan species, and free living species all have generalized pereopods.

Starfish have grasping pedicellaria and may have paxillae. If paxillae are present, the space between the paxillae may vary as the starfish moves its arms. Either arm movement, *i.e.,* reducing the space between paxillae, or pedicellaria may entrap setae or spines. Relative to the size of the propod of the pereopod, the starfish provides a flat substratum which is contacted maximally by a cylindrical propod (Fig. 1B).

Those species regularly occurring on starfish substrata exhibit a loss of setation, a loss of grasping spines, and little palmar development (Fig. 2B). These structures are secondarily derived as indicated by the advanced phylogenetic position of most species reported as occurring on starfish: four species of *Caprella, Aeginella spinosa,* and *Pariambus typicus. Caprella astericola, C. gracilior, C, unica,* and *P. typicus* almost never occur on other substrata while *A. spinosa, C. greenleyi,* and *C. linearis* only seldom are encountered on starfish. Thus, the former group should be expected to exhibit greater substratum morphological adaptations than the latter group. The pereopod of *C. greenleyi* is a form intermediate between the cosmopolitan and starfish groups of caprellids.

The morphological results of an epibiotic existence of bryozoans and gorgonians are similar, loss of spines and setae (Fig. 3C, D). However, the underlying mechanism are different. Kaufmann (1971) has shown that the bird's head avicularia of *Bugula* sieze the setae of amphipods crawling over the colony and that the avicularia are structured so that maximum force is exerted on objects 0.05 mm or less in diameter. Such a size range includes most setae on caprellids. While these avicularia provide a mechanism to retard habituation by tube-dwelling gammarids, grasping is a tactile response (Kaufmann, 1971). Therefore, setae on pereopods of caprellids would be grasped.

Gorgonians have no grasping structures, but imbeded in the coenchyme are numerous tuburculate spicules. Coenchyme is soft and penetrated easily by both setae and spines. As the gorgonian bends with the currents, the spicules of the compressed side become closer together. Setae and spines may be trapped by spicule movement. Similarly, the the ventral spine between gnathopods II on *C. equilibra* would be abraded. From preliminary experiments and observations, it is unclear if gorgonian-inhabiting caprellids initially possess spines and setae, which are subsequently abraded, or if it is a genetic ecotypic variation.

Pereopods 6 and 7 are inserted posteriorly on their respective pereonites and are six-segmented. Special attention is needed to explain insertion and size of pereopod 5. Pereopod 5 is inserted from midlength to posterior on pereonite V. Those pereopods that are inserted near the midlength may be six-segmented (as with *Luconacia incerta*), diminished in size, or reduced (as with *Mayerella* and *Perotripus*). Most genera with midlength pereopod 5 insertion have reduced pereopods. As a result, species with reduced pereopods have a modified upright stance (Fig. 2B).

All species of caprellids reported to exist free on the bottom have pereopod 5 inserted near the midlength and reduced. Contrasted with the epibiotic *Luconacia incerta,* the conclusion may be drawn that reduction of pereopod 5 is a free-living adaptation. As a result of pereopod 5 reduction, and concommitant upright stance modification, the height of elevation from the bottom is increased without an increase in body size. Visual predation may be maintained at a constant rate as body size is constant. By modifying the upright stance, a wider search and capture area for prey would result at the same body size. All caprellids that have reduced pereopod 5 lack swimming setae, *i.e.,* they are predators and scavengers (Caine, 1977). However, a similar modified upright stance and increased capture areas could be obtained by lengthening pereopod 5 rather than reducing it.

A second reduction mechanism may be that the site of pereopod 5 insertion is not suitable for variability within a genus or subfamily, but that pereopod size is. If the caprellid with a normal pereopod 5 occurs epibiotically and assumes a typical upright stance, either pereonite V will be forced close to the substratum or pereopod 5 will not encircle the substratum. Selective forces would tend to reduce pereopod 5.

A third hypothesis is that grasping the substratum with four, rather than six, pereopods is an adaptation to quiet waters. *Mayerella* is most frequently reported from quiet waters with muddy bottoms. However, *Perotripus* is found in areas exposed to extensive surge action. Pereopod 5 is reduced in both species.

It is possible that all three hypotheses explain the reduction to some extent. This would account for the widespread correlation between midlength insertion and reduction of pereopod 5. Finding a free-living representative with posteriorly inserted pereopod 5 (not as with *Pariambus typicus* which occurs on the bottom after the starfish substratum buries itself) may cast doubt on the validity of the search/capture hypothesis, but such doubt should not be formed from an epibiotic representative with reduced pereopod 5.

Further generalizations can be made regarding substratum-feeding relationships. Feeding in the Caprellidea has been seldom studied, and feeding mechanisms of relatively few species are known. However, Caine (1977) correlated the feeding of species that have been studied with the presence or absence of swimming setae. Those species with swimming setae obtain a majority of their food items by scraping or filter-feeding, while those species without swimming setae primarily rely on predation or scavenging, although scraping occurs regularly.

Caprellids usually occur in those habitats that maximize their feeding effectiveness. Within most filamentous substrata (hydroids, bryozoans, algae, etc.), no feeding mode is favored. However, gorgonians and starfish support relatively few prey species and, if observations on feeding are correct, those species with swimming setae should be more frequently encountered. *Pariambus typicus*, on the starfish *Asterias* and *Crossaster*, is a notable exception, and it is probable that *P. typicus* obtains most of its food materials by capturing mucous strands from the starfish.

Those species living free on the bottom, in contrast, are without swimming setae. Living on or within the surface layers are numerous harpacticoid copepods, ostracods, nematodes, and some gammarid amphipods upon which to prey. Flocculent material is usually silty, not detritus, and few suitable objects for scraping occur. Therefore, species existing free on the bottom should not have swimming setae.

I thank Maggie Caine and Ron Shimek for collection assistance. Friday Harbor Laboratories of the University of Washington provided research space allowing study of Pacific species; Gulf Coast Research Laboratory (Mississippi) and Ed Ball Marine Laboratory (Florida State University) provided living accommodations during the study of Gulf of Mexico species. This work was greatly enhanced by comments by Diana Laubitz on a preliminary draft and by Craig Staude on a late draft.

Summary

1. Caprellid amphipods occur epibiotically or free on the bottom. There are four general types of substrata and caprellids have distinctive morphological adaptations to each substratum type.

2. Those caprellids without specific substratum preferences have primative propod structure including proximal grasping spines and distinct palm development.

3. Starfish epibionts have cylindrical propods and both setae and spines are reduced. Bryozoan and gorgonian epibionts have morphological adaptations similar to starfish epibionts, but the selective forces for the parallelism differ between the two groups.

4. Caprellids existing free on the bottom have reduced pereopods 5, which result in a modified upright stance. The modified upright stance allows a greater prey search area without an increase in body size.

5. Various substratum types support different arrays of available food for the caprellids. Gorgonians and starfish generally support scraping/filter-feeding species while those species regularly occurring free on the bottom are predators. No feeding type is favored on most filamentous substrata.

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MECHANISMS OF WOOD DIGESTION IN THE SHIPWORM *BANKIA GOULDI* BARTSCH: ENZYME DEGRADATION OF CELLULOSES, HEMICELLULOSES, AND WOOD CELL WALLS

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Wood in the marine environment is rapidly invaded by bivalve mollusks and isopod crustaceans (Jones and Eltringham, 1971). The bivalves most active in the destruction of sound wood belong to the family Teredinidae (Turner, 1966), commonly called shipworms or teredos. These organisms grow and tunnel rapidly through hard, dry wood while avoiding softer, more rotten regions (Board, 1970). Wood fragments are scraped from the anterior end of the tunnel and enter the digestive tract where they are processed by two structures unique to wood boring mollusks: a very large wood storing caecum and digestive diverticula specialized for wood phagocytosis (Sigerfoos, 1907; Potts, 1923; Morton, 1970).

The unique habitat and digestive tract structure of the Teredinidae suggest two important questions: do shipworms digest wood, and if so, by what mechanisms? Several attempts have been made to demonstrate *in vitro* enzymes active in the digestion of cellulose and wood. Enzymes degrading purified celluloses have been detected (Boynton and Miller, 1927; Greenfield and Lane, 1953; Deschamps, 1953; Nair, 1955a, b, 1956a, b, c; Crosby and Reid, 1971), but in those cases where the cellulose was in a crystalline structure similar to its inferred structure in wood cell walls, the level of activity was extremely low (Kadota, 1959; Boynton and Miller, 1927; Cutter and Rosenberg, 1972; Rosenberg and Cutter, 1973). Production of soluble sugars from ground wood using teredinid extracts has also been reported (Boynton and Miller, 1927; Kadota, 1959).

Wood is not composed solely of cellulose; it is a complex material consisting of cellulose, hemicelluloses, and lignin (Meier, 1964; Northcote, 1972). Cellulose, 40% by weight of the wood cell wall, is a high molecular weight unbranched polymer of β-1,4-D-glucopyranose; the polymers are aggregated into crystalline fibrils (Gardner and Blackwell, 1974a, b; Blackwell and Kolpak, 1975; Stoekmann, 1972). This *native crystalline cellulose* is an anhydrous, water-insoluble material of great stability (Ranby, 1969). The hemicelluloses, about 30% by weight of gymnosperm wood, are a diverse group of non-crystalline hydrophilic polysaccharides partly adsorbed to the cellulose fibrils (Timell, 1964, 1965). The unique properties of wood are conferred by the lignin component (30% of gymonosperm wood), a highly inert and insoluble resin-like compound encrusting the carbohydrates and protecting them from solvent and solute interaction (Pew and Weyna, 1962; Ranby, 1969; Stone, Scallan, Donefer and Ahlgren, 1969; Kirk, 1971).

Some species of fungi and bacteria, together with some insects, gastropod mollusks and ruminant mammals are able to survive and grow on native crystalline cellulose as a sole carbon source, while other organisms are restricted to non-crystalline cellulose derivatives or forms of cellulose with a more labile crystal structure (Reese, Siu, and Levinson, 1950; Reese and Levinson, 1952; Wood, 1969). In fungi, a complement of diverse hydrolytic enzymes degrades native crystalline cellulose to glucose (Hajny and Reese, 1969). Non-native celluloses are labile to some of these same enzymes. Enzymes active on various β-1,4-glucans, including native cellulose, are termed β-1,4-glucanases (Reese, Maguire and Parrish, 1968; Barras, Moore and Stone, 1969) and may have exo- or endo-activity. In the case of insoluble cellulose particles such as those entering the digestive tract of *Bankia gouldi,* digestive enzymes must produce significant solubilization of the particles if cellulose is to play a nutritionally significant role. The ultimate products of exo- and endo- β-1,4-glucanases will be cellotetraose, cellotriose, and cellobiose, which will be hydrolyzed to glucose by β-glucosidase.

The research reported here was designed to clarify the digestive capabilities of hydrolytic enzymes in extracts of the digestive tract of the teredinid bivalve *Bankia gouldi* Bartsch, assayed as activity producing soluble sugars, principally glucose, from cellulose and wood. The significance of the observed cellulolytic activity for wood digestion was evaluated by comparing *Bankia* tissue extracts to better characterized commercial preparations of cellulolytic enzymes and interpreting the results in the light of our current understanding of wood and cellulose structure. Enzymes with high activity on native crystalline celluloses could not be demonstrated, but β-1,4-glucanase and β-glucosidase activity was readily observed.

MATERIALS AND METHODS

Specimens of *Bankia gouldi* were collected by immersing pine blocks, purchased from a local lumber yard, under the dock at the Duke University Marine Laboratory, Beaufort, North Carolina. After 1–4 weeks in the sea, the blocks were moved to aerated standing seawater aquaria. The organisms were removed from the wood and the digestive tracts were cut free of the surrounding tissue. The tracts were routinely homogenized in 0.1 M sodium chloride with either a blender or a sonicator, depending on the amount of tissue. A variety of homogenization and extraction media were tested. The homogenates were incubated for up to 12 hours at both 0° C and 37° C in the following solutions: distilled water; 0.1, 0.5, 1.0 M sodium chloride; 0.01 M disodium EDTA; 0.001 M mercaptoethanol; 0.1% Triton X-100; 0.1 M sodium acetate buffers, pH 5.0; 0.1 M sodium borate buffer, pH 9.0. Bacterial growth was inhibited either with sodium azide (0.003 M) or with toluene. In no case was any enhancement of enzyme activity seen over that observed with the 0.1 M sodium chloride.

Homogenates were either dialyzed in acetylated dialysis tubing against 0.02 M sodium phosphate buffer pH 8.0, or centrifuged at 100,000 × *g,* and the supernatant ultrafiltered under pressure on an Amicon PM-10 ultrafilter with the same buffer. A constant problem in this study was obtaining sufficient quantities of tissue extract to characterize the enzyme activity. Shipworms do not actively tunnel throughout the year and they are difficult to remove from wood. As a result, different batches of tissue extract were used at various phases of the study.

To assess the potential nutritional significance of any observed enzyme activity in the *Bankia gouldi* extract, some parallel experiments were run with cellulose degrading enzymes from other sources. Commercial preparations from better-characterized sources were chosen: "Cellulase" from the fungus *Trichoderma viride* (Worthington Biochemical Co.); "β-glucuronidase" from the gastropod mollusk *Helix pomatia* (Sigma Chemical Co.); and "Cellulase" from the fungus *Aspergillus niger* (Sigma Chemical Co.). These commercial preparations were dissolved in or diluted with distilled water, centrifuged at 100,000 × *g* and washed on a PM-10 ultrafilter with 0.1 M sodium acetate buffer, pH 5.0.

Substrates for enzyme assays

Cellulose can exist in a variety of forms, and the lability of cellulose polymers to degradation by hydrolytic enzymes is a function of the degree of crystallinity and the precise crystal structure of the substrate (Norkrans, 1967; Rautela and King, 1968; Cowling and Brown, 1969). In addition, as part of the wood cell wall, cellulose is complexed with other potentially protective materials. No single cellulose substrate can provide adequate information on the activity of β-1,4-glucanases. Seven cellulosic substrates were therefore selected to study the influence of crystallinity, and the presence of other molecules on the capabilities of the digestive enzymes.

Milled wood (MW) was prepared from sawdust from the wood used to collect specimens of *Bankia gouldi*. The sawdust was ground in a Wiley mill to pass through a 60 mesh, yielding a fine powder with a particle size distribution somewhat larger than *Bankia* gut contents (based on microscopical observation). *Holocellulose,* wood with the lignin fraction removed, was prepared by the standard sodium chlorite method (Browning, 1967). The resulting white powder was washed thoroughly with water and air-dried. *Alpha-cellulose*, a partially purified wood cellulose, was also prepared by standard procedures, involving extraction with potassium hydroxide (Browning, 1967).

Microcrystalline cellulose (MC) was purchased from Sigma (Sigmacell Type 19). This highly crystalline material is produced by controlled acid hydrolysis of native crystalline cellulose (Halliwell, 1966; Battista, 1971). *Swollen microcystalline cellulose* (SMC), also called Walseth cellulose (Walseth, 1952a), was prepared by suspending MC in 85% cold phosphoric acid (Stone *et al.*, 1969). This treatment increases the hydration of the polymers, separates fibrils, and produces a partial transition to an alternate, folded chain crystal structure (Warwicker, 1971; Stone *et al.*, 1969). *Carboxymethyl cellulose* (CMC) was purchased (Sigma). This is a substituted, non-crystalline and fully water soluble β-1,4-glucan (Eriksson, 1969). *Cellobiose*, the disaccharide unit of cellulose, was also purchased (P–L Laboratories).

Enzyme assay methods

Cellulose and wood degradation were measured as production of soluble products from the substrate. Tissue extracts were assayed for solubilizing activity on the various substrates at 37° C; the choice of temperature was arbitrary. Standard assay conditions were: 0.05 M sodium phosphate buffer, pH 6.4 or 7.0; 0.003 M sodium azide; and substrate concentrations as indicated in Table I. Substrate-free and protein-free controls were run for all experiments. Incubations were terminated by heating to 100° C, and the residual particulate material removed by centrifugation. A sample of the supernatant was removed for the determination of soluble products. Total reducing sugars were measured with the Somogyi-Nelson technique (Nelson, 1944; Somogyi, 1952) using glucose as a standard; results are expressed as micromoles of glucose-equivalents per milliliter. Glucose was measured enzymatically with glucose oxidase, horseradish peroxidase, and o-dianisidine (Sigma) (Okuda and Miwa, 1973). Protein concentration in tissue extracts was determined by the Lowry technique. One unit (U) of enzyme activity was defined as the production of one micromole of soluble product per minute. *Specific activity* was defined as units per milligram protein (U/mg protein).

The influence of pH on initial rate of hydrolysis assayed as glucose production was studied using 0.05 M sodium citrate and sodium phosphate buffers. Enzyme stability as a function of pH in the absence of substrate was measured by incubating dialyzed *Bankia* supernatant in the same buffers for 24 hours at 4° C and then assaying under the standard conditions at pH 7.0.

Assays over time were run by removing samples from incubations at the indicated intervals and determining the products present. The commercial enzyme preparations were assayed in 0.05 M sodium acetate buffer, pH 5.0, with 0.003 M sodium azide and processed as above.

RESULTS

Homogenates of *Bankia gouldi* digestive tracts produced glucose from all cellulose substrates tested (Table I). The specific activity, standardized for unit weight of substrate, is an index to the relative lability of the substrates to enzyme degradation. SMC is clearly the most labile substrate while MC is the least labile. The lability of MW appears to be even lower (Table II); but since MW is only about 70% carbohydrate (Dean, 1976), the specific activity per milligram carbohydrate is 0.008. All the glucose-producing enzyme activity was soluble and remained in the supernatant after centrifugation.

Preliminary characterization of the glucose producing activity was carried out using dialyzed supernatant material as a source of enzyme. The optimum pH for

TABLE I

Specific activities of dialyzed Bankia *homogenate on cellulose substrates. Specific activity is in terms of units* (U) *per milligram protein, where one unit is 1.0 micromole of glucose produced per minute from the standard substrate concentration. Standard assay conditions, pH 6.4; 10 minute incubations.*

Substrate	Abbreviation	Standard Concentration (mg/ml)	Specific activity per mg substrate ± standard deviation
Microcrystalline cellulose	MC	5	0.27 ± 0.09
Swollen MC	SMC	1.7	14.90 ± 1.50
Carboxymethyl-cellulose	CMC	5	7.70 ± 0.40
Milled wood	MW	10	—
Cellobiose	—	3.4 (1 micromole/ml)	554.00 ± 44.00

TABLE II

Rates of glucose production from cellulose substrates by different enzyme systems. Rate is expressed as activity units (U); *one unit is one micromole of glucose produced per minute from the standard substrate concentrations. Specific activity is units per mg protein. Initial rate was measured over the first 15 minutes of the reaction; final rate over the last 2 hours. Data is from experiments shown in Figure 1.*

Enzyme source	Substrate	Rate $\times 10^{-3}$ (U/mg substrate)	Specific activity $\times 10^{-3}$ (U/mg protein)	Time period for rate calculation
Trichoderma	SMC	5.53	19.882	Final
	MC	0.12	0.488	Final
	MW	0.02	0.083	Final
Helix	SMC	4.47	16.000	Final
	MC	0.54	0.190	Final
	MW	0.15	0.054	Final
Aspergillus	SMC	27.82	46.410	Initial
	MC	0.01	0.016	Final
	MW	0.003	0.005	Final
Bankia	SMC	12.65	23.235	Initial
	MC	0.014	0.024	Final
	MW	0.005	0.009	Final

glucose production from SMC was found to be between pH 6.0 and 7.0, which was also the region of maximum enzyme stability in the absence of substrate. The maximum rate of glucose production was observed at 40° C, when tested over a range of 20–50° C. The detection of β-1,4-glucanase activity, necessary for SMC degradation, is in part a function of β-glucosidase. The stability of the latter enzyme was found to be sensitive to the buffer used, but had a broad stability over the range of pH 4.0 to 8.0, sufficiently active to hydrolyze any cellobiose produced from SMC hydrolysis. The rate of β-glucosidase activity was maximum at 50° C, suggesting that β-glucosidase is not directly responsible for SMC degradation.

The rate of glucose production from cellulose and cellobiose showed a linear increase when increasing amounts of tissue extract protein were added, as is characteristic for enzymes. The substrate concentration was also related linearly to the rate of glucose production from all substrates. The enzyme activity could not be saturated with substrate using MC or CMC up to levels of 70 mg substrate per mg tissue extract protein. However, SMC saturated the available enzymes at a much lower level, 9.6 mg SMC per mg extract protein; cellobiose saturated β-glucosidase activity at still lower levels. The relationship between the rate of glucose production and the substrate concentration, when standardized for unit weight of protein added, also provides an index to relative substrate lability. The failure of CMC and SMC to saturate available enzymes even at very high substrate levels shows them to be relatively inert to the enzymes involved.

The activity of *Bankia gouldi* tissue homogenate through time on SMC, MC, and MW was compared to the activity of commercial enzyme preparations. With the *Bankia* extract, the substrates were saturated with enzyme at the beginning of the incubation period, and β-glucosidase levels were sufficiently in excess so that all soluble products of substrate hydrolysis would appear as glucose.

Enzymes from *Trichoderma viride* and *Helix pomatia* produced glucose at a

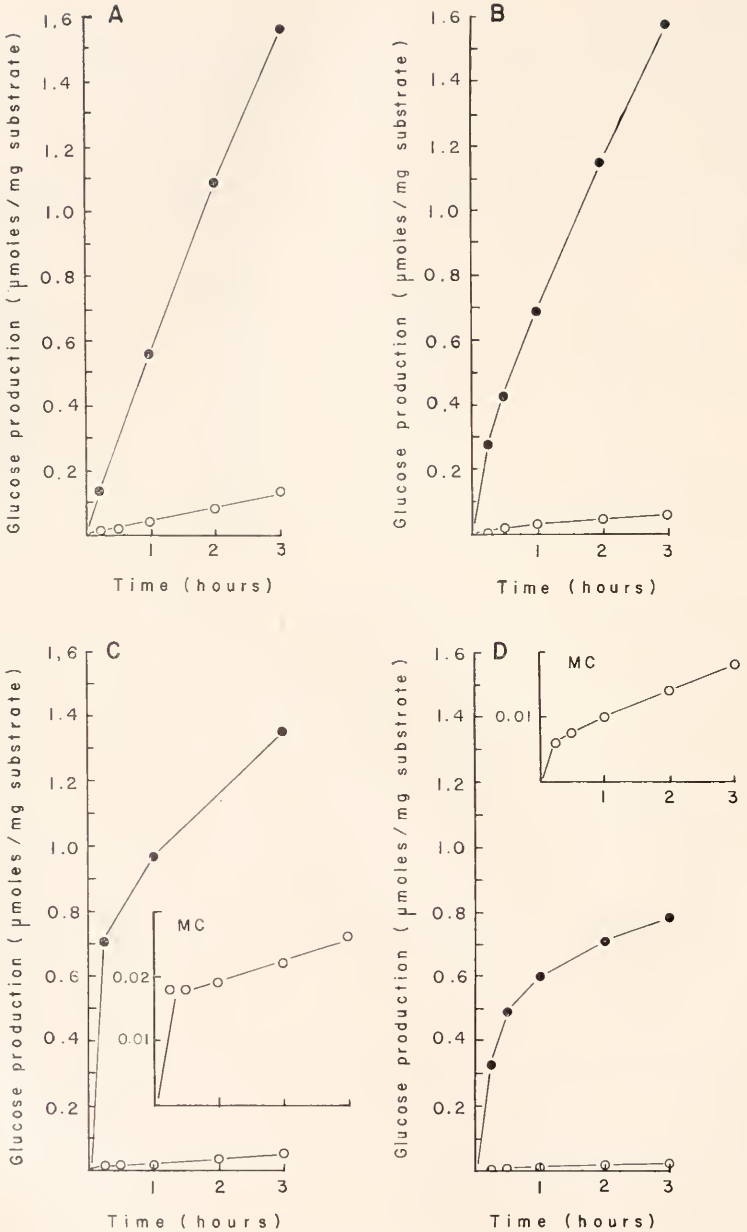

FIGURE 1. Glucose production over a three hour period from cellulose substrates by four enzyme preparations. Standard substrate concentrations at 37° C: A, *Trichoderma viride* enzymes, assayed in 0.05 M sodium acetate buffer, pH 5.0; B, *Helix pomatia* enzymes, assayed in 0.05 M sodium citrate-sodium phosphate buffer, pH 5.0; C, *Aspergillus niger* enzymes, assayed in 0.05 M sodium acetate buffer, pH 5.0; and D, *Bankia gouldi* dialyzed homogenate, assayed in 0.05 M sodium phosphate buffer, pH 6.4. Solid circles represent SMC; open circles, MC.

nearly linear rate through time from SMC, MC, and MW (Figs. 1A, 1B; Table II). This pattern is typical for organisms capable of growth on native crystalline cellulose; the linear rate persists for days *in vitro* (Halliwell, 1966; Dean, unpublished). Both MC and MW were attacked at significantly slower rates, as is consistent with the relative substrate lability. The pattern of activity on these two native substrates is not the same for the two species, however. When the activity is expressed in terms of glucose produced per milligram of carbohydrate, *Helix* enzymes show a MW to MC ratio of 0.46, as compared to a ratio of 0.26 for *Trichoderma viride*. This can be interpreted as showing MW to be relatively more labile to *Helix* enzymes.

The results observed with *Aspergillus* and *Bankia* enzymes show a quite different pattern of activity over time (Figs. 1C, 1D; Table II). Glucose production from SMC is initially rapid and then gradually declines. A similar burst seen with MC and MW is followed by a low, but constant rate of glucose production. Although the activity of these two preparations on SMC was higher than that observed with *Trichoderma* and *Helix*, the rates of glucose production on native crystalline cellulose (MC) were an order of magnitude lower than with the latter two preparations. *Bankia gouldi* extract did show a higher level of activity on MC relative to SMC than did *Aspergillus* enzymes (Table II).

The declining rate of glucose production over a three hour period from SMC by *Bankia* enzymes can be explained as a result of gradual enzyme inactivation (proteases are probably present in the crude extracts), product inhibition, or the disappearance of a more labile fraction of the substrate to yield an inert residue. The results in Figure 2 test for these alternatives. After incubating SMC with *Bankia*

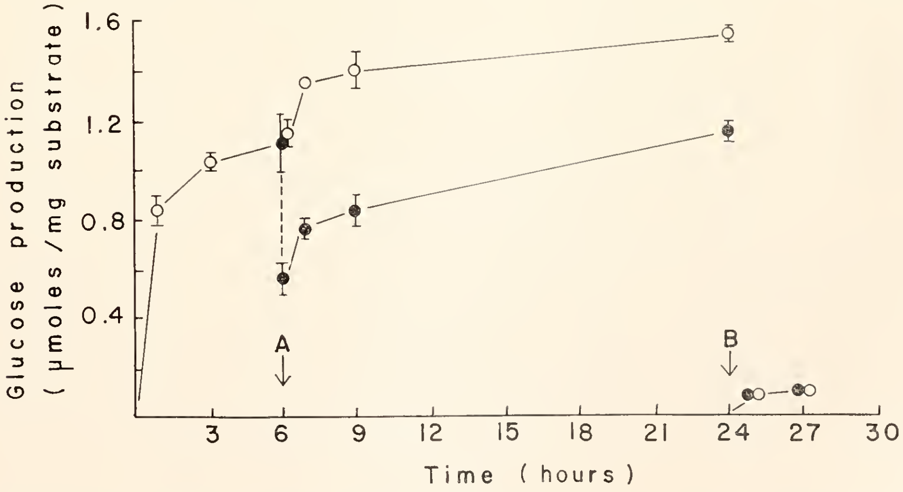

FIGURE 2. Glucose production over time from SMC by *B. gouldi* dialyzed homogenate, standard assay conditions, pH 7.0. Vertical bars indicate standard deviation. At A, the reaction was halted by lowering temperature to 0° C. At B, the reaction was halted by heating the same tubes to 100° C. Residual substrate was washed until glucose-free, and then re-incubated with fresh homogenate. Solid circles represent medium diluted 1:1 with buffer, no substrate removed, and fresh homogenated added; open circles, fresh homogenate added.

extracts for six hours, fresh extract was added, producing a second burst of glucose production whether or not soluble products had been removed. When the substrate was then washed by centrifugation after more extended incubation, and fresh *Bankia* extract added, the subsequent rate of glucose production is comparable to that seen on untreated MC (Fig. 1).

The lability of wood and wood fractions to *Bankia* enzymes was also measured, since this material is the natural substrate for digestive enzymes. The MW used

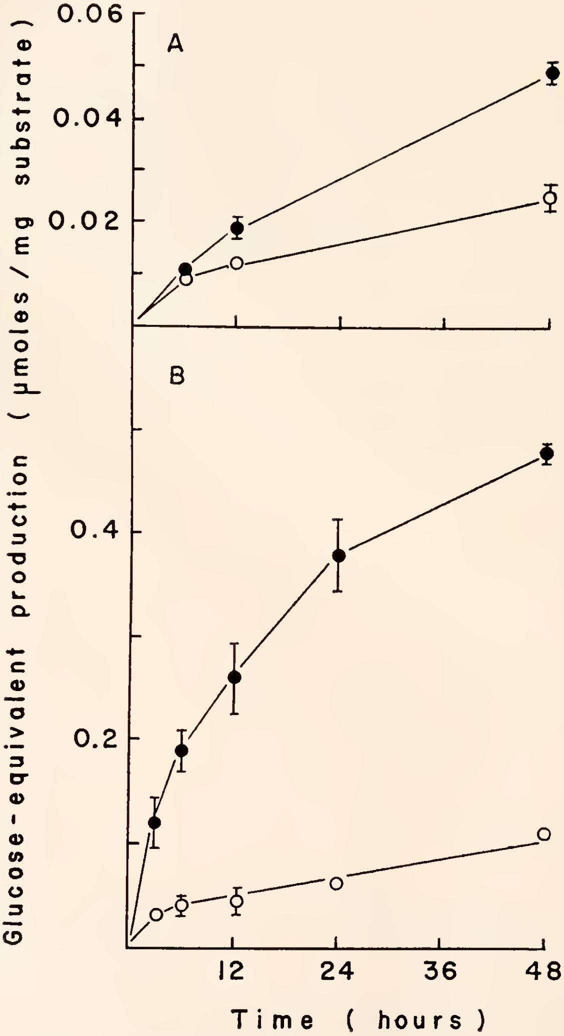

FIGURE 3. Production of soluble sugars over time by *B. gouldi* dialyzed homogenate: standard assay conditions, pH 7.0. A, MW, 37 mg substrate/mg protein; B, delignified MW (holocellulose), 37 mg substrate/mg protein. Solid circles represent total reducing sugars; open circles, glucose.

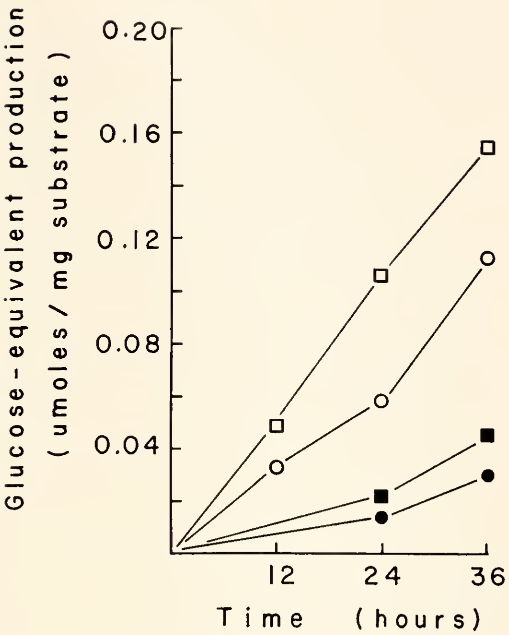

FIGURE 4. Production of soluble sugars by *B. gouldi* dialyzed homogenate from milled wood (squares) and α-Cellulose (circles). The substrate (ordinate) refers to mg carbohydrate in the substrate particles. Both glucose (*closed symbols*) and total reducing sugar (*open symbols*) production are shown.

in these studies is about 70% carbohydrate (Dean, 1976) of which about 60% is native cellulose (Meier, 1964). The observed rate of glucose production from this material by *Bankia* enzymes is consistent with the crystallinity of wood cellulose (Fig. 3A). The higher rate of total reducing sugar production from MW contrasts with results for purified celluloses, where rates of reducing sugar and glucose production are identical, indicating glucose to be the major product.

Removing the lignin fraction from wood greatly enhances the lability of MW to *Bankia* enzymes (Fig. 3B). If the hemicellulose fraction is extracted from the delignified wood, the α-cellulose residue shows greatly decreased lability, here expressed as sugar production per mg carbohydrate in the substrate (Fig. 4). The *Trichoderma* preparation, on the other hand, showed activity on α-cellulose substantially higher than that seen on MW. This α-cellulose residue consists of native crystalline cellulose and glucomannan hemicellulose (Browning, 1967).

DISCUSSION

Available evidence strongly suggests that wood makes an important contribution to the energy requirements of *Bankia gouldi* and other species of wood boring shipworms. The ingestion of wood particles and the uniquely specialized digestive tract argue for an important role. The maintenance of large glycogen reserves (30% of dry tissue weight) in *Lyrodus pedicellatus* is dependent on a supply of wood

(Lane, Posner, and Greenfield, 1952), and shipworms removed from the wood die a few days after emptying the caecum, even if provided with phytoplankton (Turner and Johnson, 1971). This is particularly significant in the light of recent demonstrations that adult specimens of *Bankia gouldi* are capable of suspension feeding (Dean and Back, 1977). Lazier (1924) noted progressive loss of haemotoxylin-positive material from wood fragments during passage through the gut of *Teredo navalis*, which can be interpreted as the loss of the hemicellulose fraction (Freundlich and Robards, 1974). Fecal pellet analyses with *Teredo navalis* (Dore and Miller, 1923) and *Bankia gouldi* (Dean, 1975, 1976) indicate substantial loss of the carbohydrate fraction of wood, including all that is accessible to water soluble enzymes.

The mechanism of wood digestion in shipworms has proved to be as elusive and difficult to study as it is in other organisms, due to present uncertainty about the structure of wood itself. Although the chemistry of most of the cell wall components has been well characterized, the behavior of these components within the wall has not (Timell, 1964, 1965; Northcote, 1972; Stoekmann, 1972). The interactions of these fractions are crucial in determining the lability of the material to organisms. In the case of *Bankia gouldi*, it must be asked what relevance any observed enzyme activity has to the requirements of the organism as it tunnels through wood. Therefore, a brief review of current concepts of cell wall structure and degradative mechanisms is necessary.

Wood is a composite material consisting of the cell walls of dead cells. The bulk of wood is composed of fibrous cells characterized by greatly thickened walls which are the main supportive element of trees (Mark, 1967). In softwoods (gymnosperms), as used in this study, the cell is the tracheid; in hardwoods (angiosperms), the fiber cell (Meylan and Butterfield, 1972). These cells may be considered the basic unit of wood.

The wood cell wall has a laminar structure (Mark, 1967; Meylan and Butterfield, 1972). The primary wall is the first to be laid down and is characteristic of all plant cells (Albersheim, 1976). Most of the cell mass in wood, however, is in the secondary wall, characterized by a high density of cellulose fibrils wound helically around the wall and encrusted with matrix material (Mark, 1967). This secondary wall can be looked at as a single cross-linked molecule, resolvable into three phases, corresponding to three chemical fractions: a crystalline phase of cellulose fibrils; a hydrophilic phase of hemicelluloses coating the surface of the crystalline fibrils; and a hydrophobic phase, continuous throughout the wall, in which the other phases are embedded, and consisting of lignin (Dean, 1975, 1976).

The primary emphasis in research on wood digestion in teredinid shipworms and other wood decaying organisms has been on the cellulose fraction. This emphasis is probably a result of the economic importance of wood cellulose as well as the unique problems posed by the structure of the material and the mechanisms of its degradation. It is clear that wood cellulose is in the form of crystalline fibrils (Mark, 1967; Heyn, 1969; Preston, 1971). The best supported model for isolated fibrils appears to be a rigid array of antiparallel polymers linked by hydrogen bonds (Gardner and Blackwell, 1974a, b; Blackwell and Kolpak, 1975) with randomly distributed disruptions and imperfections (Muhlethaler, 1969). Some evidence suggests cellulose in wood can be considered paracrystalline and distinct in structure from isolated cellulose in ways that would affect its lability to digestive enzymes (Stoekmann, 1972; King, 1968).

In electron micrographs, the crystalline fibrils appear unstained but do have a cortex 10 to 20 Å thick with a high affinity for dyes (Heyn, 1969; Freundlich and Robards, 1974). Since hemicelluloses are known to adsorb strongly to cellulose fibrils (Northcote, 1972), this cortex is assumed to be a layer of hydrophilic hemicelluloses. This very polydisperse group of polymers (Timell, 1964, 1965; Northcote, 1972) has adhesive properties and probably holds the cellulose fibrils together prior to the deposition of lignin (Mark, 1967; Albersheim, 1976).

The crystalline and hydrophilic phases together make up a system of "sticky" fibers wound around the cell; these fibers are embedded in lignin just as the glass fibers in fiberglass are embedded in resin. Lignin is a phenolic polymer forming a three-dimensional, cross-linked gel throughout the wall (Bolker and Brenner, 1970) and is covalently and hydrogen bonded to the hemicelluloses (Northcote, 1972). The hydrophobic nature of lignin (Pew and Wewna, 1962; Kirk, 1971; Wayman and Obiaga, 1974) makes it the critical component in stabilizing the wall because it governs the permeability of the wall to water and the hydration of the hemicellulose fraction (Ranby, 1969; Stone *et al.*, 1969). Lignin also accounts for the high compressive strength of wood (Mark, 1967).

An organism digesting wood cell walls probably will have evolved mechanisms for two critical steps in wood digestion: first, delignification, or exposing the crystalline and hydrophobic phases to enzymes; and secondly, depolymerizing and solubilizing the cell wall constituents themselves. The actual mechanisms by which wood ingesting invertebrates carry out these processes are very poorly understood. Model mechanisms can be developed from the large literature on wood and cellulose–degrading microorganisms, especially fungi (Reese, 1963; Norkrans, 1967; Hajny and Reese, 1969; Kirk, 1973). The major weakness of these studies is that most have dealt with the degradation of isolated cell wall components, usually cellulose, or their derivatives. Frequently the organisms studied are themselves unable to digest whole wood (Merrill, 1966; Wood, 1969). These models do, however, present testable starting points for studying wood-digesting invertebrate organisms. But wood is preeminently a complex macromolecular system, and when this system can be dealt with as a whole, surprises are certain to emerge.

Although some fungi can remove lignin enzymatically (Kirk, 1971), the only mechanism of delignification of demonstrable significance to teredinid shipworms is mechanical disruption of the cell wall. Prolonged milling of wood under various conditions with vibratory or rolling ball mills allows a separation of lignin and carbohydrate (Kirk, 1971; Wayman and Obiaga, 1974). Even short milling times greatly increase the digestibility of the wall by fungal enzymes (Pew and Weyna, 1962; Kirk, 1971). The mechanism probably involves fracturing the lignin matrix away from the carbohydrate phases, exposing them to the medium (Wayman and Obiaga, 1974). One would expect wood digestibility to increase as particle size decreases, as has been shown in soil decay studies (Neal, Bollen, and Lu, 1965).

Hydrophobic and water soluble polysaccharides such as the hemicelluloses are degraded to monosaccharides by the synergistic activity of exo- and endo-hydrolases (Dekker and Richards, 1976). The degradation of cellulose is more problematic. The crystalline fibrils are insoluble in water and display minimal solvent interaction

(Ranby, 1969). Most interior glycosidic linkages are constrained from interacting readily with enzymes, and the hydrogen bonds prevent deformation of the glycosidic linkages, which seems to be an important feature of the catalytic mechanism of at least one endo-hydrolase (Dunn and Bruice, 1973). While the decomposition of cellulose is a biologically unusual process, it is not yet possible to explain how it is unusual at the biochemical level.

As with hemicellulose digestion, the decomposition of native crystalline cellulose at a rate sufficient to support the growth of an organism apparently requires the synergistic activity of several enzymes, each of which may show some solubilizing activity. The classical model for the mechanism of native cellulose degradation supposes that there is a fundamental qualitative difference between the enzyme complement necessary for the degradation of the native substrate and the enzymes required for swollen or soluble cellulose decomposition (Reese *et al.,* 1950). In biological terms, this concept is valid, since some fungi can grow on all celluloses while others are restricted to the more labile forms (Reese and Levinson, 1952; Wood, 1969). This model has proved elusive when pursued biochemically.

Over the past decade, a great deal of effort has gone into the isolation of enzymes from fungal and bacterial sources active on native cellulose. Considerable success has been achieved with the extracellular enzymes released by the fungus *Trichoderma viride* and the wood-rotting fungus *Sporotrichum pulverulentum,* but the results do not fit well with the original model proposed by Reese *et al.* (1950). However, several enzymes have been isolated, which when acting together, extensively degrade native crystalline cellulose to soluble products. Endo-β-1,4-glucanases randomly hydrolyze interior glycosidic bonds, producing chain ends to be attacked by an exo-β-1,4-glucanase producing cellobiose as a principle product (Berghem and Pettersson, 1973; Berghem, Pettersson, and Axio-Fredriksson, 1975, 1976; Eriksson and Pettersson, 1975a, b; Streamer, Eriksson, and Pettersson, 1975). In all cellulolytic microorganisms studied, β-glucosidase catalyzed cellobiose and cellodextrin hydrolysis as the final step in glucose production from cellulose (Berghem and Pettersson, 1974).

The results reported here clearly demonstrate that native crystalline cellulose is relatively resistant to degradation by all enzyme preparations when compared to other forms of cellulose. The patterns of glucose production over time (Fig. 1) are similar for *Trichoderma viride* and *Helix pomatia:* enzymes degrade MC and SMC at a uniform rate through time. *Trichoderma viride* is a soil fungus which can be cultured on native crystalline cellulose as a sole carbon source (Mandels and Reese, 1964), although it cannot grow on wood (Merrill, 1966; Stone *et al.,* 1969). The gastropod mollusk *Helix pomatia* has been shown, like *Trichoderma,* to produce enzymes with high activity on native crystalline cellulose and other plant polysaccharides (Holden, Pirie and Tracey, 1950; Holden and Tracey, 1950; Myers and Northcote, 1959; Koopmans, 1970). That such a similar pattern may be seen in such phylogenetically distant species suggests that the observed pattern may be interpreted as typical for organisms digesting native cellulose at a nutritionally significant rate.

The level of activity of both *Aspergillus niger* and *Bankia gouldi* enzymes on MC was very low but measurable (Fig. 1; Table II), reflecting the very low lability of the substrate. In the case of *Bankia* homogenates, this rate was the maximum possible with the stated substrate concentration, since the rate of MC solubilization was independent of enzyme concentration; adding additional MC would lead to enhanced glucose production. Since the reaction was substrate limited, the low rate must be a function of a very limited number of sites on the MC particle labile to enzyme attack.

The very high initial rate of glucose production from SMC by both *Bankia gouldi* and *Aspergillus niger* enzyme preparations reflects the enhanced lability of the substrate. The phosphoric acid treatment, which swells the fibrils and alters the crystal lattice (Stone *et al.*, 1969; Warwicker, 1971), probably increases the accessibility of interior glycoside linkages to digestive enzymes. The decline in rate was shown to be in part a function of enzyme inactivation (not including product inhibition), but also a result of decreasing substrate lability (Fig. 2). As the labile fraction is removed, a residual native crystalline core may remain (Walseth, 1952a, b); alternatively, the cellulose chains may rearrange themselves into the more resistant native structure as a result of the degradation (Cowling and Brown, 1969; Stoekmann, 1972). This same pattern has been reported for other preparations (Norkrans, 1950; Walseth, 1952b; Cowling and Brown, 1969).

Aspergillus niger is a fungus called "pseudocellulolytic" by Wood (1969) because of its inability to grow on native crystalline cellulose, while it can grow on swollen and soluble celluloses. The extracellular enzymes, including endo-β-1,4-glucanases (Hurst, Nielson, Sullivan and Shepherd, 1977), have previously been shown to have limited activity on the native substrate but high activity on other β-1,4-glucans (Walseth, 1952a, b; Wood, 1969). The similarity in activity pattern of *Bankia gouldi* enzymes to enzymes from *Aspergillus niger* certainly implies that this level of native crystalline cellulose degradation is insufficient to support the growth and survival of *Bankia gouldi,* and insufficient to explain the extent of cellulose digestion estimated from fecal pellet analysis (Dean, 1975, 1976). The high level of activity on SMC unambiguously demonstrates β-1,4-glucanase activity and implies that both exo- and endo-enzymes are present in *Bankia gouldi* (Berghem and Pettersson, 1973; Berghem *et al.*, 1976). Further conclusions await the isolation of the individual enzymes.

Such enzymes are primarily interesting for their potential role in wood degradation. The lability of wood and wood fractions to *Bankia gouldi* tissue extracts is consistent with the model for the structure of the wood cell wall and with the results obtained with pure cellulose substrates. While the production of glucose and reducing sugars from milled wood (MW) was low, it was greatly enhanced by delignification, emphasizing the protective effect of the hydrophobic phase. Alpha-cellulose, on the other hand, was highly resistant to the *Bankia gouldi* enzymes, as expected for a native crystalline substrate. Since total reducing sugar production was higher than glucose production for all wood substrates tested, enzymes were digesting the hemicelluloses in wood. Even α-cellulose remains contaminated with glucomannan hemicelluloses (Browning, 1967).

If studies on other teredinid species are reinterpreted in the light of concepts presented here, they are consistent with the results on the digestive capabilities of *Bankia gouldi* tissue extracts. β-1,4-glucanase activity ("cellulase") has been reported for *Teredo navalis* (Kadota, 1959; Deschamps, 1953), *Bankia setacea* (Crosby and Reid, 1971), *Bankia indica* (*carinata*) (Nair, 1955a, b, 1956a, b, c), and *Teredo* (*Lyrodus*) *pedicellatus* (Greenfield and Lane, 1953; Lane and Greenfield, 1952, 1953; Greenfield, 1955). β-glucosidase activity has been described for *Bankia setacea* and appears identical to the activity reported here (Liu and Walden, 1969). *Bankia setacea* (Crosby and Reid, 1971), *Bankia indica* (*carinata*) (Nair, 1955a, b, 1956a, b, c), and *Teredo navalis* (Boynton and Miller, 1927; Kadota, 1959) have been reported to produce reducing sugars from ground wood; enhancement of production after delignification was seen with *Teredo navalis* enzymes (Kadota, 1959).

The β-1,4-glucanase activity reported for *Teredo* (*Lyrodus*) *pedicellatus* (Greenfield and Lane, 1953) showed essentially the same pH behavior seen here with *Bankia gouldi*. A partial purification of a *Lyrodus* enzyme was achieved with an affinity method involving CMC, but the activity was not further characterized (Greenfield, 1955). The *Teredo navalis* enzyme activity also showed similarities to my results with *Bankia gouldi*. However, a bimodal pH-activity curve with "cellulose" as the substrate with optima at pH 5.6 and 6.6 has been reported for *Teredo navalis* (Kadota, 1959). A similar bimodal result has more recently been reported for this species but with optima at 4.6 and 6.1 (Cutter and Rosenberg, 1972; Rosenberg and Cutter, 1973).

In all species of teredinid shipworms investigated, the activity of tissue extracts on native crystalline cellulose was low (Kadota, 1959; Nair, 1955a, b, 1956a, b, c; Boynton and Miller, 1927; Cutter and Rosenberg, 1972; Rosenberg and Cutter, 1973). The only quantitative data is for *Teredo navalis,* where a maximum rate of glucose production from MC of 2×10^{-6} U/mg MC (as calculated from their figures) was recorded (Rosenberg and Cutter, 1973). This activity is more than an order of magnitude lower than the low activity reported here for *Bankia* (Tables I, II). Significant solubilization of native crystalline cellulose *in vitro* has therefore not been demonstrated with any teredinid shipworm, despite claims to the contrary.

In conclusion, the results for *in vitro* enzyme activity from *Bankia gouldi* will be discussed in terms of the mechanisms of feeding and digestion in this and other shipworm species. *Bankia gouldi,* in tunneling through wood, fractures the cell wall into fine particles, ranging down to 10 μ or less (Plate 18, Fig. 1 in Dore and Miller, 1923). This fracturing probably disrupts the lignin matrix and exposes a significant fraction of cell wall polysaccharide, including cellulose. Once ingested, these particles may be stored in the caecum, where they may be exposed to extracellular digestive enzymes. Some portion of these particles may also enter the specialized digestive diverticula and be phagocytosed (Potts, 1923; Morton, 1970), with possible exposure to lysosomal digestive enzymes (Owen, 1974). Hydrolysis of cellobiose and other oligosaccharides by β-glucosidase and other glycosidases may be extra or intracellular.

Since the activity of the hemicellulolytic enzymes producing reducing sugars from wood can be detected at pH 6.0 to 7.0, which is in the normal range for bivalve digestive tracts (Owen, 1974), they may be extracellular enzymes active in the stomach and caecum. The extent to which these enzymes can produce soluble products from wood particles will be limited by the lignin fraction and will be a function of the particle size (Pew and Weyna, 1962; Stone *et al.,* 1969; Neal *et al.,* 1965; Wayman and Obiaga, 1974).

The β-1,4-glucanase activity reported here for *Bankia* is probably active in breaking β-1,4-glycosidic linkages in hemicelluloses, but its role in the digestion of wood cellulose remains uncertain. The failure to demonstrate enzyme activity producing soluble products from native crystalline cellulose at a nutritionally significant rate remains a problem. There are several possible explanations. Fracturing the cell walls and digesting the hemicelluloses should expose a portion of the cellulose to enzyme attack. While these fibrils are crystalline, they may be less crystalline, and therefore more labile, than commercial MC. Rautela and King (1968) emphasize the importance of precise crystal structure to enzyme lability, while King (1968) found that isolated celluloses were less labile to fungal enzymes than intact cell wall cellulose. The possibly paracrystalline nature of the fibrils when within the wall has been emphasized by others (Muhlethaler, 1969; Stoekmann, 1972). The β-1,4-glucanases may in fact solubilize ingested wood particle cellulose to an extent necessary to explain the cellulose content of fecal pellets (Dore and Miller, 1923; Dean, 1976).

A second explanation is that the failure to demonstrate the expected enzyme activity is an experimental artifact. Enzymes may be lost during the extraction procedure. Even in whole homogenates, the enzymes may remain bound to wood fragments in the caecum; some β-1,4-glucanases from *Trichoderma* show strong binding to crystalline celluloses (Mandels, Kostick and Parizek, 1971). Alternatively, the enzymes may simply be inactive under the chosen extraction and assay procedures. If so, they are very different from their fungal counterparts.

In the event that lysosomal enzymes are responsible for cellulose digestion, it is difficult to predict a mechanism. Hydrolases, perhaps active at acid pH, may be involved; an exciting possibility is that non-hydrolytic mechanisms play an important role, and the principle products of digestion are not glucose or reducing sugars. The coupling of cellobiose oxidation to the reduction of lignin quinones is a possibility (Westermark and Eriksson, 1974a, b, 1975), and any cellulose degradation would not be detected by measuring glucose production. Alternatively, other non-hydrolytic reactions, inactive under the assay conditions, may be necessary for maximum hydrolysis (Eriksson, Pettersson and Westermark, 1974). Cellulose digestion may therefore involve as yet undescribed oxidative reactions that are characteristic of intact, metabolically active cells. Elucidating the mechanism may therefore involve formidable technical problems in cell fractionation of the phagocytic cells.

This work was carried out as part of a doctoral dissertation in the Department of Zoology, Duke University, Durham, North Carolina. Support was provided by the Office of Naval Research (Contract No. NR 104–194 to J. D. Costlow and R. C. Dean; Contract No. NR 104–981 to R. C. Dean).

SUMMARY

Tissue extracts from the digestive tract of the wood-boring bivalve *Bankia gouldi* were tested for their ability to degrade celluloses and wood to soluble sugars.

Tissue extracts showed high levels of glucose production from cellobiose, demonstrating the presence of β-glucosidase.

The crystal structure of cellulose determines its lability to enzymes. Microcrystalline cellulose (MC) was used as an analogue to native crystalline cellulose in wood cell walls. Swollen microcrystalline cellulose (SMC) and carboxymethyl cellulose (CMC) are more labile celluloses. *Bankia goudi* extracts showed maximum activity on SMC and minimum activity on MC, when assayed as glucose production.

When assayed over time, glucose production from SMC gradually declined as a function of enzyme inactivation and decreasing substrate lability. These results are interpreted as showing the removal of a cellulose fraction of altered crystal structure, leaving the more inert native crystalline fraction.

Activity of *Bankia gouldi* extracts over time was compared to commercial cellulose-digesting enzyme preparations. The pattern of activity closely resembles that of *Aspergillus niger,* a fungus unable to grow on native crystalline cellulose. The pattern is very different from that seen with *Trichoderma viride* and *Helix pomatia* enzymes, characterized as true cellulose decomposing systems.

The production of glucose from ground wood by *Bankia gouldi* extracts is consistent with the crystallinity of the substrate and the results for purified cellulose. A higher level of production of reducing sugars other than glucose indicates the digestion of wood hemicelluloses.

Digestibility of wood hemicelluloses is limited by the lignin fraction in the wood cell wall.

The experimental results were interpreted in terms of a model for the molecular architecture of the wood cell wall. The cellulose is present as crystalline fibrils coated with hydrophilic hemicellulose polymers. These coated fibrils are embedded in a hydrophobic lignin matrix. The digestibility of wood is a function of polymer accessibility and lability to digestive enzymes.

The enzymes demonstrated *in vitro* in *Bankia gouldi* tissue extracts are β-1,4-glucanases with limited activity on native crystalline cellulose, which they cannot digest at a nutritionally significant rate. The mechanism of cellulose digestion by shipworms remains an unsolved problem.

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GUT ULTRASTRUCTURE AND DIGESTIVE PHYSIOLOGY OF TWO MARINE NEMATODES, *CHROMADORINA GERMANICA* (BÜTSCHLI, 1874) AND *DIPLOLAIMELLA* SP.[1]

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Relative to other phyla, the Nematoda encompasses large numbers of species living in virtually every known benthic habitat. There have been several approaches to understanding the apparent coexistence of large numbers of nematode species. In studies of field populations, variables such as temperature, oxygen concentration, and salinity have been related to the abundance and distribution of nematode species (*e.g.*, review by McIntyre, 1969; Pamatmat, 1968; Tietjen, 1969, 1971; Coull, 1970; Meyers, Hopper, and Cefalu, 1970; Warwick and Buchanan, 1970; Warwick, 1971; Ward, 1973; Wieser and Schiemer, 1977). Other approaches have used a laboratory setting to determine nematode requirements for or tolerances to environmental variables (*e.g.*, Tietjen, Lee, Rullman, Greengart, and Trompeter, 1970; Gerlach and Schrage, 1971; 1972; Tietjen and Lee, 1972; Hopper, Fell, and Cefalu, 1973; Ott and Schiemer, 1973; Schiemer, and Duncan, 1974).

In shallow benthic environments, a large array of potential foods for nematodes exist (Lee, McEnery, Kennedy, and Rubin, 1975; Fenchel, 1969). The heterogeneous distributions of different food species may account for much of the variety and large numbers of nematode species found in the benthos, assuming that different nematode species are feeding on different foods. Wieser (1953, 1960) theorized about the likelihood of nematodes being feeding specialists—living in similar microenvironments but existing on different foods in the benthos. He grouped nematodes into feeding types according to the size of the buccal cavity and the extent and type of buccal armature. He reasoned that differences in nematode diets could be due to different mouthparts and, therefore, to differing abilities to ingest various food species. Recently, tracer feeding techniques have been used to demonstrate that selective feeding may occur in several nematodes (Tietjen, Lee, Rullman, Greengart, and Trompeter, 1970; Tietjen and Lee, 1973, 1977).

Seemingly slight differences in the digestive physiologies of marine nematode species may reflect adaptations to different diets. It is generally assumed that nematode digestion, aside from the process of ingestion, conforms to a similar pattern throughout the taxon—free-living soil, freshwater, and marine, as well as parasitic forms (reviewed by Lee, 1965; Bird, 1971; Lee and Atkinson 1976). The digestive tract is a straight tube differentiated into buccal cavity, esophagus, esophageal-intestinal valve, intestine, and rectum. There are three esophageal gland cells that empty their contents into the esophageal lumen. The esophagus is usually a pumping organ which forces food into the intestine. The intestine consists of a single layer of cells. Digestion is extracellular in nearly all species, although an intracellular phase does occur in some (Colam, 1971a, b).

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The purpose of this study was to compare the digestive physiologies of two species of marine nematodes, including the microanatomy of their digestive tracts, and to compare their abilities to ingest and digest different foods. Ultimately, this information might help to explain the large numbers of nematode species coexisting in the benthos.

MATERIALS AND METHODS

Nematode culturing

Two nematode species, originally isolated from a salt marsh at Towd Point, Southampton, Long Island, New York, were cultured for this study: *Diplolaimella* sp. and *Chromadorina germanica* Bütschli 1874. Nematodes were maintained in 60 mm plastic petri dishes with an overlay of 10 ml of 0.45 μm HA millipore-filtered sea water (mfsw) with a salinity of 26‰. A mixture of an antimycotic/antibiotic (penicillin 100 U/ml, fungizone 0.25 mcg/ml, streptomycin 100 mcg/ml) was added to the medium. The cultures were maintained at 20°–24° C under a light regime of 18 L : 6 D. Approximately 0.1 ml of suspended food organisms, at a concentration of 10^8 cells/ml, were added to each dish. These foods were obtained from stock cultures of algae grown on "S" medium (Lee, Stone, Muller, Rullman, and McEnery, 1970). The seawater overlay was changed once a week. Nematode cultures were transferred about once a month. In addition to the added foods, other species of algae and bacteria grew in the culture dishes, and thus cultures were considered to be agnotobiotic.

Electron microscopy

Whole specimens of *Diplolaimella* sp. were fixed for 2 hr at 4° C in 3% glutaraldehyde in millipore-filtered seawater (mfsw). Specimens were rinsed briefly in mfsw and post-fixed in 2% osmium tetroxide in mfsw for 2 hr at 4° C. Whole specimens of *C. germanica* were fixed for 1 hr at 4° C in 3% glutaraldehyde in mfsw; but immediately after the fixative was added, the cuticle was cut open by cutting off the anterior end of the nematode. Specimens were then processed as above.

Specimens of both species were dehydrated with acetone using the sequence 30% acetone for 10 min at 4° C, 70% acetone overnight or up to two days at −11° C, 90% acetone for 15 min at 4° C, three changes of 100% acetone 15 min each at 20° C. Specimens were placed in a mixture of 1 : 1 acetone to Spurr's embedding medium (1969), placed under vacuum for 45 min, and left uncapped in the hood for an additional 45 min. Finally, specimens were embedded in fresh Spurr's and polymerized at 70° C for 24 hr.

Primary fixation was carried out in small glass wells which were in turn placed in petri dishes to prevent evaporation. Fixative was removed carefully by

Pasteur pipette and several drops of 1% agar in mfsw at 47° C were added to the well. This was immediately mixed with a metal spatula to suspend the nematodes in the agar matrix. When the agar had hardened, it was transferred to a glass vial filled with the appropriate rinse solution. Processing continued with nematodes suspended in this agar for all steps up to and including embedding. To embed, small chunks of agar containing specimens were cut out with a razor and forceps under a dissecting scope and placed in flat embedding trays.

Light gold to silver to gray sections were prepared with an LKB ultramicrotome using a diamond knife. Sections were mounted on uncoated 200-mesh or 300-mesh copper grids (Polysciences, Inc.), stained with saturated aqueous uranyl acetate followed by 3% lead stain (Sato 1967), and viewed with a Philips 300 Electron Microscope at 80 kv. Kodak Electron Image plates were used for taking pictures.

Feeding experiments

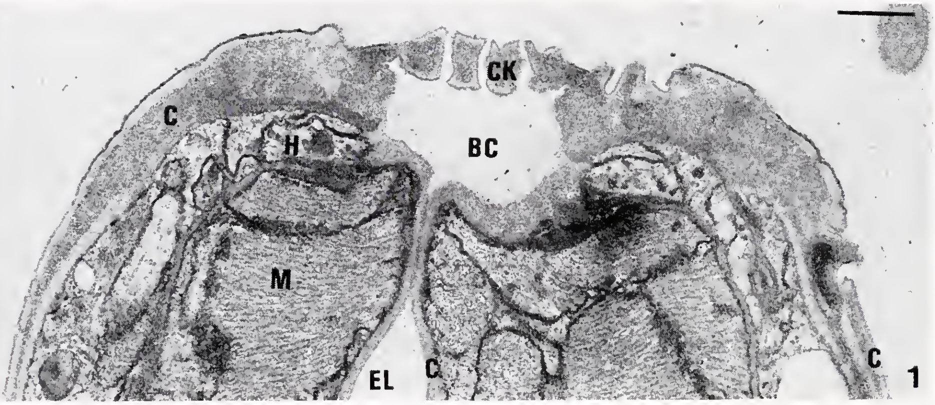
For this series of experiments, nematodes were fed a variety of different potential food species to test nematode ability to ingest and digest each food. About 30 to 50 adult nematodes were gently pipetted into sterile petri dishes, taking care to transfer very little of the medium into the new dish. Individual microfloral species were harvested from stock cultures by centrifugation, washed and resuspended in 0.2 ml mfsw. The food cells chosen for these experiments included six algal and two bacterial species and they were fed to nematodes as single foods. For controls, nematodes were pipetted into dishes without food. Dishes were examined and counted daily for live nematodes for up to five days. Some nematodes were removed after one to two days and processed for the study of gut contents. Food cells were processed along with the nematodes.

Histochemistry and cytochemistry

Specimens were fixed in 3% formaldehyde in sea water for 2 hr. Specimens were washed briefly in mfsw and incubated for nonspecific esterase activity using the indoxyl acetate method of Holt (1958) and arylamidase activity (Burstone and Folk, 1956). Controls included specimens incubated without the substrate and after heat inactivation at 100° C for 5 min. Whole mounts were examined with the light microscope.

For visualization at the electron microscope level, nematodes were fixed in 1% glutaraldehyde in sea water for 15 min, washed with mfsw 1 min, incubated for acid phosphatase (Barka and Anderson, 1962) at room temperature for 15 min, rinsed in mfsw, and then fixed again in 3% glutaraldehyde in sea water for 30 min. Routine procedures were followed for post-fixation, dehydration, and embedding.

For visualization of surface proteins in the fibrillar matrix lining the luminal borders of the intestinal cells of *Diplolaimella* sp., specimens were fixed routinely and dehydrated and embedded without post-fixation. The test for glycoproteins was made on sections collected on uncoated grids and immersed for 5 min in 0.5% phosphotungstic acid in 0.1 N hydrochloric acid, pH 1.2 (Quintarelli, Bellocci, and Geremia, 1973).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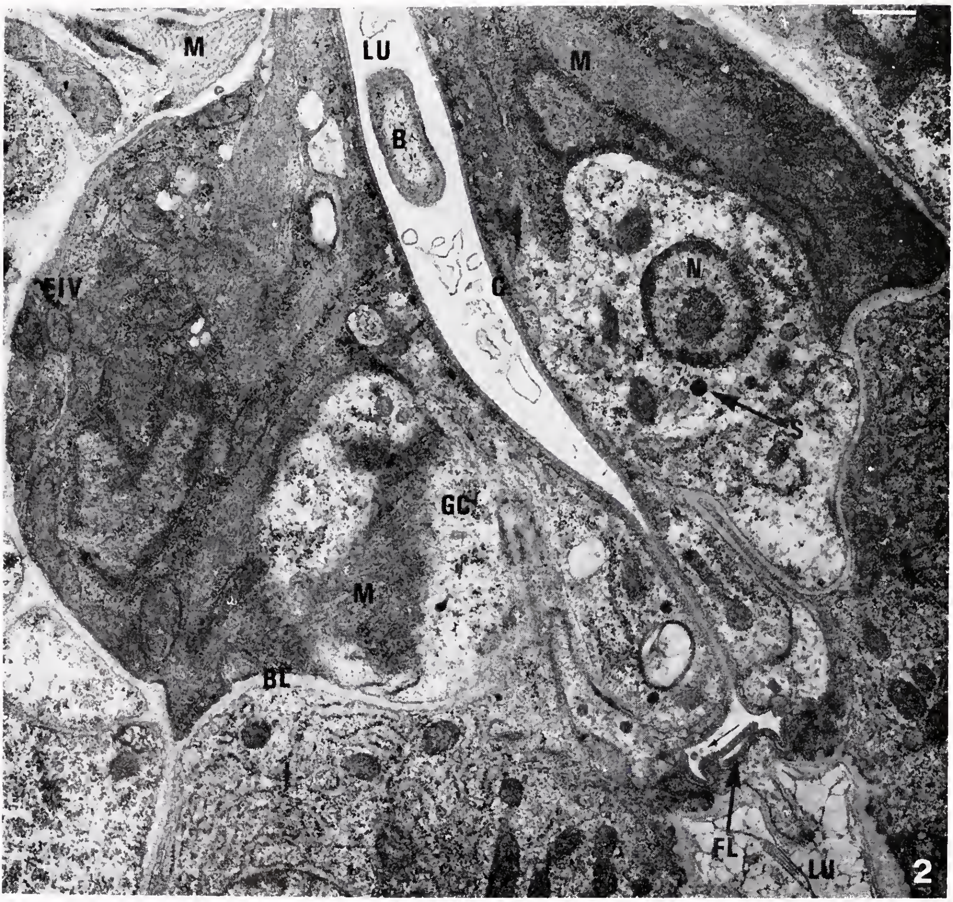
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FIGURE 1. An obliquely longitudinal section through the anterior end of *Diplolaimella* sp. showing the buccal cavity and esophageal lumen lined with cuticle and transverse muscles. BC =

RESULTS

Ultrastructure of the digestive tract: Diplolaimella sp.

Diplolaimella sp. has a typical nematode digestive tract, consisting of buccal cavity, muscular esophagus, esophageal-intestinal valve, intestine, and rectum. The buccal cavity is a circular opening approximately 1 μm in diameter surrounded by 12 rounded cuticular knobs (Fig. 1). It leads into the triradiate esophagus, which is cylindrical in shape anteriorly, but forms a bulb-like swelling at its posterior end. It is a typical nematode esophagus with no unusual features.

Between the esophagus and the beginning of the intestine is a cuticular-lined esophageal-intestinal valve consisting of several cells (Fig. 2). The valve is separated from the esophagus by a small band of encircling muscle filaments. The valve also has sets of muscle filaments which run radially from the cuticle lining the valve lumen to the external wall of the valve. Just posterior to the muscle cells are several secretory cells or gland-like cells which form the rest of the valve. These secretory cells protrude anteriorly into the valve muscle bands for part of their length. They contain membrane-bounded inclusions filled with fibrillar and electron-transparent materials or electron-dense materials. The muscles of the valve may, because of the position of these secretory cells, squeeze secretions out of the cells into the valve lumen when the muscles contract. No ducts were found, but the posterior valve lumen usually contained several dense granules than could have originated from these secretory cells.

The intestine is formed by a single layer of epithelial cells. In any cross-section only two cells circumscribe the intestinal lumen. These cells have very little stratification and exhibit cell organelles scattered at random throughout the cell.

The first two anterior sets of cells are typical secretory cells (Fig. 2). They each have a well-developed and extensive, rough, endoplasmic reticulum. There are several Golgi complexes. A few electron-dense cell inclusions are scattered in the distal regions of the cells. The microvilli are approximately 0.12 μm in diameter and 0.5 μm in length. They are closely packed at the cell surface bordering the intestinal lumen. Microfilaments are oriented along the longitudinal axes of the microvilli. A thin basal lamina bounds the intestinal cells at their external borders.

The remaining intestinal cells are all similar in structure and are not specialized along the length of the intestine (Fig. 3). Each cell has a well-developed, rough, endoplasmic reticulum and large numbers of mitochondria which are distributed throughout the cell. Occasionally, a multivesicular body occurs in these cells. Much of the cell volume is taken up by cell inclusions which have been described in the literature as "pigment granules." These are single membrane-bounded spheres

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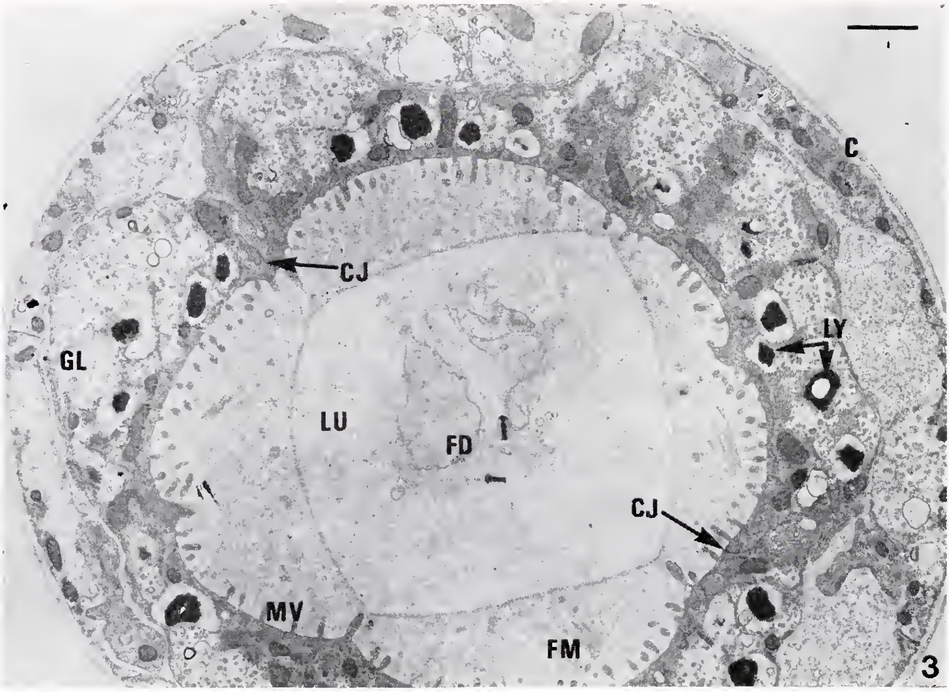
buccal cavity, C = cuticle, CK = cuticular knobs, EL = esophageal lumen, H = hypodermis, M = muscle. Bar is 0.5 μm.

FIGURE 2. Semi-longitudinal section of *Diplolaimella* sp. showing part of the muscle band that encircles the valve lumen, the valve's muscular bulb, and its gland–like cells. The anterior intestine is also visible. The secretory products are very electron dense. The small arrow points to secretory-like material in the valve lumen. The flaps separating the valve lumen from the intestinal lumen are also shown. B = bacterial cell, BL = basal lamina, C = cuticle, EIV = esophageal-intestinal valve, FL = flaps, GC = gland-like cell, I = intestine, LU = lumen, M = muscle, N = nucleus, S = secretory product. Bar is 0.5 μm.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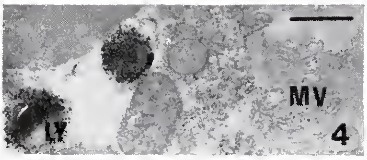

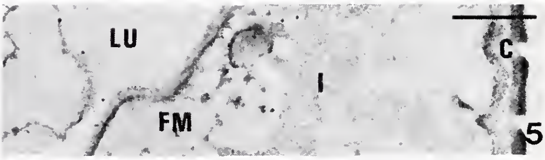

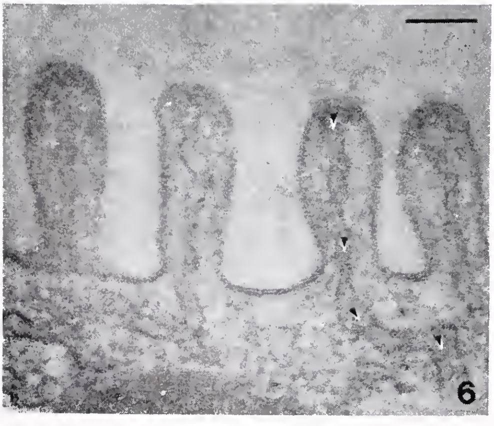

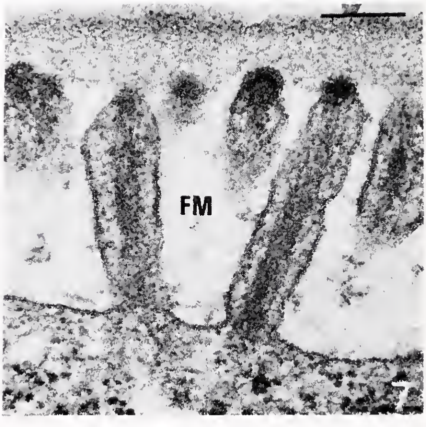

FIGURE 3. Typical transverse section through two intestinal cells of an adult female *Diplolaimella* sp. Unusual microvilli are at the four "corners" of the "square" outlined by the

containing very electron-dense materials. Some of these granules are positive for acid phosphatase, showing that some are actually lysosomes (Fig. 4). Less electron-dense granules are found peripheral to the lysosomes. They are not acid-phosphatase positive, but may represent an earlier stage in the production of the lysosomes. Because these intestinal cells lack any secretory apparatus, they are most probably absorptive cells.

The entire intestinal lumen is coated along the luminal surfaces of the cells with a well-organized fibrillar matrix (*e.g.*, Fig. 3). In transverse section it sometimes appears to be formed into four ridges that protrude into the lumen and four valleys or rays. Within the fibrillar matrix three zones of varying electron density could be distinguished. There is a narrow dense zone at the lumen-matrix interface which occasionally resembles a membrane but is not a typical unit membrane. There is a less dense homogeneously fibrillar matrix just under this zone. The third zone, closest to the intestinal cell membrane, occupies the largest volume of the fibrillar matrix. This zone of the matrix is fairly uniformly diffuse but within it are electron-dense fibrillar globules, parallel sheets of fibrils, and membrane-bounded vesicles (Fig. 3). The vesicles appear to contain the same fibrillar material which is found outside them. These vesicles are most numerous at the very beginning of the intestine, and sometimes occur in the matrix lining the rest of the intestinal cells. Their origin may be the anterior secretory cells of the intestine. The vesicles were sometimes found between the microvilli at their bases, throughout the inner matrix zone, and especially collected just beneath the middle zone. They may play a role in the maintenance and renewal of the fibrillar matrix.

The chemical composition of the matrix is unknown. There was staining of the narrow zone of the lumen-matrix interface, some small areas within the third zone of the fibrillar matrix, and the external cuticle with phosphotungstic acid applied to nonosmicated thin sections (Fig 5). It is possible, then, that the fibrillar matrix has proteins similar to those found in the cuticle.

The luminal surfaces of these intestinal cells are modified to form the very unusual microvilli (Fig. 6) which were sometimes found in the anterior four intestinal cells. Each microvillus had a cisterna along its central axis that was 0.02–0.04 μm in diameter and continuous with the endoplasmic reticulum. The cisterna ended blindly near the tip of the microvillus. The microvilli are regularly spaced but not as closely packed as in the anterior cells. In well-fed specimens, these

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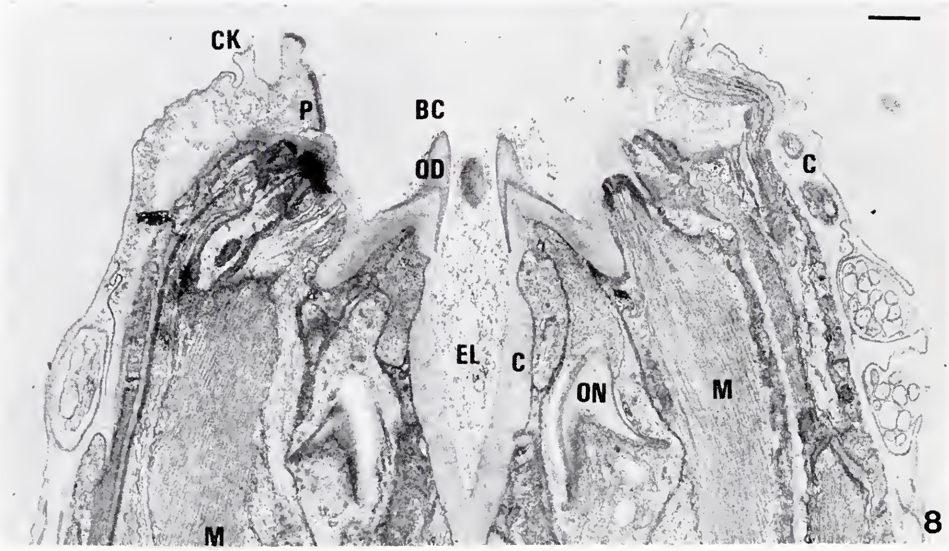
fibrillar matrix. C = cuticle, CJ = cell junction, FD = food debris, FM = fibrillar matrix, GL = glycogen, LU = lumen, LY = lysosome, MV = microvilli. Bar is 1 μm.

FIGURE 4. An oblique section through the intestine of *Diplolaimella* sp. showing lead deposits in the "pigment granules" from the acid phosphatase reaction, not counterstained. LY = lysosome, MV = microvilli. Bar is 0.5 μm.

FIGURE 5. Part of a transverse section through the intestine of *Diplolaimella* sp. that was stained with phosphotungstic acid at pH 1.2. Both the cuticle and luminal border of the fibrillar matrix show electron density (as does some food debris in the lumen); FM = fibrillar matrix, LU = lumen, I = intestine. Bar is 0.5 μm.

FIGURE 6. Detail of the microvilli of the intestinal cells of *Diplolaimella* sp. showing the cisterna of the microvillus continuous with the cisterna of the endoplasmic reticulum. The arrowheads trace this continuum. Bar is 0.1 μm.

FIGURE 7. Detail of the unusual microvilli associated with the fibrillar matrix of *Diplolaimella* sp. FM = fibrillar matrix. Bar is 0.2 μm.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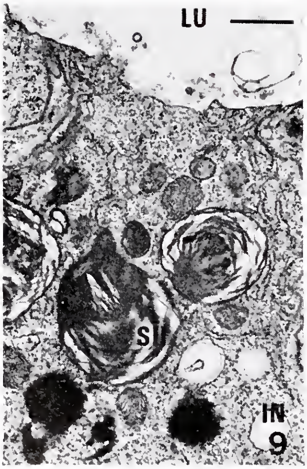

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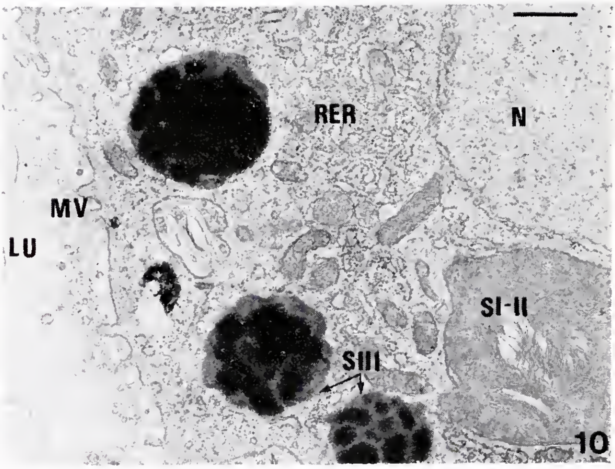
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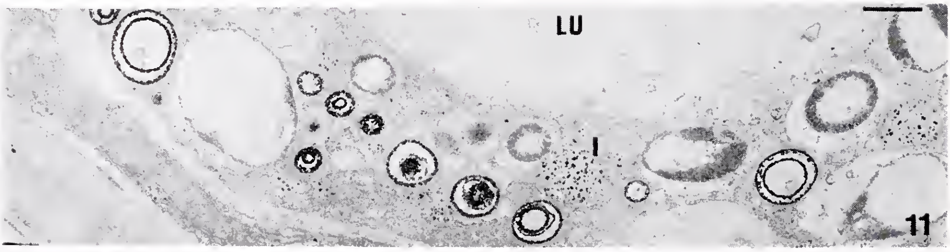

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Figure 8. Longitudinal section through the anterior end of *C. germanica* showing the pads forming the mouth, the two sets of teeth (odontia and onchia), and longitudinal muscles. BC =

cells lose their usual appearance of a clearly defined cisterna along the central axis of each microvillus. Instead, the microvilli appear swollen and more electron-transparent.

There are specialized microvilli located immediately adjacent to the intercellular junction between the two circumluminal cells and in the area of the cell membrane equidistant between the two cell junctions. These four sets of longitudinally aligned microvilli are filled with microfilaments running along the central axis of each. The tips of these microvilli have a very electron-dense cap just under the plasma membrane of the microvillus (Fig. 7). Trailing from the tip of each specialized microvillus is dense fibrillar material which is continuous with the middle zone of the fibrillar matrix. The microfilaments in these microvilli may anchor and stabilize the middle zone of the matrix. These microvilli may function to maintain the physical integrity of the matrix.

Ultrastructure of the digestive tract: Chromadorina germanica

C. germanica has a larger mouth than *Diplolaimella* sp. (Fig. 8). The mouth measures approximately 2.9 μm in diameter. An unusual set of small pads, analogous to lips, surround the buccal cavity. Just lateral to the pads, the external cuticle is sculptured with knob-like projections. The pads, together with these knobs, may help to hold and position food particles for subsequent ingestion.

The entire buccal cavity is lined with a dense, unsculptured cuticle. The cuticle is modified to form three electron-dense sharp pointed teeth, or odontia, protruding from the base of the buccal cavity and surrounding the entrance to the esophagus (Fig. 8). Muscle fibers run posteriorly from their attachment points at the cuticle lining the buccal cavity to the esophageal musculature. These longitudinally-running muscle fibers may help to temporarily enlarge the buccal cavity and expose the teeth. This contrasts with *Diplolaimella* sp., which has neither pads nor odontia and where all muscles around the buccal cavity run transversally.

The buccal cavity opens into the esophagus just posterior to the three teeth. Another set of three teeth, or onchia, which are pointed elaborations of cuticle, are embedded in the anterior end of the esophagus (Fig. 8).

While the esophagus is generally similar to that of *Diplolaimella* sp., it differs from the latter in several respects. The dorsal esophageal gland cell empties into the anterior portion of the esophageal lumen somewhat posterior to that of *Diplolaimella* sp. and the bulb of the esophagus of *C. germanica* is larger and wider than that of *Diplolaimella* sp. In contrast with the latter, each muscle filament is

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buccal cavity, C = cuticle, CK = cuticular knob, EL = esophageal lumen, M = muscle, OD = odontia, ON = onchia, P = pad. Bar is 0.5 μm.

FIGURE 9. Transverse section through the intestine of *C. germanica* showing some of the different cell inclusions, including the electron-transparent inclusion. IN = inclusion, LU = lumen, SII = stage II inclusion. Bar is 0.5 μm.

FIGURE 10. Transverse section through the intestine of *C. germanica* showing some of the different cell inclusions. LU = lumen, MV = microvilli, N = nucleus, RER = rough endoplasmic reticulum, SI–II = Stage I-II inclusion, SIII = Stage III inclusion. Bar is 0.5 μm.

FIGURE 11. Transverse section of the intestine of *C. germanica* showing lead deposits, in a variety of cell inclusions, from the acid phosphatase reaction, not counterstained. I = intestine, LU = lumen. Bar is 0.5 μm.

approximately 6 μm, or twice the length, in the bulb region. Thus, *C. germanica* can exert more pressure on potential food.

The esophagus and intestine are separated by several cells forming a valve-like structure. These cells are typical protein-synthesizing cells.

In contrast to *Diplolaimella* sp., six cells encircled the intestinal lumen of *C. germanica* in any cross-section. Only one cell type was distinguishable with no regional specialization along the length of the intestine.

All intestinal cells have few, short, irregularly-spaced microvilli. The cells show transmembrane activity, possibly endocytosis, as indicated by small invaginations of the cell membrane that were coated on their outer leaflets with fibrillar or fuzzy material that varies in thickness and extends from the outer leaflet of the microvillar membrane into the lumen.

All intestinal cells appeared to have a potential for protein synthesis; each has typical protein-synthesizing features: extensive rough endoplasmic reticulum with distended cisternae, scattered Golgi complexes, and a prominent nucleus containing finely diffuse chromosomal material bounded by a nuclear membrane studded with ribosomes. This high potential degree of synthetic activity contrasts with the majority of the intestinal cells of *Diplolaimella* sp., which are absorptive.

A variety of membrane-bounded cell inclusions were randomly distributed in the intestinal cells throughout the length of the intestine. It is impossible to tell, however, from ultrastructure alone, if these inclusions represent different stages in the processing of digestive material. It is possible, however, to reconstruct a probable chronological development of these inclusions.

The largest sized inclusion body, Stage I (my terminology), contains small vesicular-shaped profiles and some membrane-like materials. Stage II is characterized by whorls of lamellae (Fig. 9). Transition stages between I and II are common (Fig. 10). Stage III is characterized by several large, dense, spherical granules enclosed by a single membrane (Fig. 10). The last stage, Stage IV, results from the possible coalescing of these granules to form inclusions which are sometimes uniformly very dense and sometimes grainy (Fig. 9). At times these inclusions are not fully infiltrated by the plastic embedding material. Stage IV appears similar to the lysosomal inclusions (pigment granules) found in *Diplolaimella* sp. These inclusions are sometimes observed in the process of being extruded into the gut lumen, thereby representing the final stage in the development of these inclusions. Another possibility is that these inclusions represent separate stages in two different processes. The Stage I inclusion is similar to an autophagosome and may be digesting worn-out cell parts. Stage II may be the residual body of this process. Or, Stage II may include the breakdown of cell membranes and may therefore function in cell membrane conservation. The Stages III and IV would still represent steps in the lysosomal digestion of food molecules.

Several other types of cytoplasmic inclusions have occasionally been observed in the intestinal cells: multivesicular bodies and vacuolar inclusions bodies are often associated with a golgi complex. Because the latter type of inclusion and some, but not all, of the stage IV inclusions are acid-phosphatase positive they can be considered lysosomes (Fig. 11).

There were occasional dying intestinal cells being sloughed off into the lumen throughout the length of the intestine. The cytoplasm of each dying cell exhibits a gradient of density, in contrast to that of typical intestinal cells in *C. germanica,* with the least dense region of the cell protruding furthest into the intestinal lumen. This region consists of some microtubules and membrane-like material scattered throughout an electron-transparent matrix. The region of the cell closer to the cell's basal lamina contains a cytoplasm of nearly normal density with intact organelles or, in the case of cells in advanced stages of dying, with swollen mitochondria, a few distended cell inclusions, and short strips of rough endoplasmic reticulum. Significantly, there are no secretory products in these cells. This phenomenon may represent a type of holocrine secretion whereby the cell dies immediately after secreting its packaged enzymes into the lumen.

Ingestion and digestion of foods

Digestion in Diplolaimella *sp.* Reconstruction of the major events of digestion was deduced by following the natural food as it was broken down and by studying the digestive tracts of specimens in different nutritional states.

Food was generally ingested as whole, intact cells by the pumping action of the esophagus. The dorsal esophageal gland secretions coat the food when it enters the buccal cavity and then the subventral esophageal gland secretions coat the food cells as they move down into the esophageal lumen. There was no histochemical evidence that these secretions were enzymatic in nature, although these gland cells have been shown to secrete hydrolytic enzymes in other species (*e.g.,* Lee, 1965). Tests for esterase and arylamidase activities were negative in this region. These glands may be secreting components of one or more digestive enzyme which only become activated when mixed together in the lumen or on the food. The food may be further coated by the secretions from the cells of the esophageal-intestinal valve, although these cells were also negative for hydrolytic enzymes.

Morphological evidence for the initial chemical breakdown of the food was found in the intestinal lumen. Both intact and partially broken down food cells were distributed throughout the lumen (Figs. 12, 13). Esterases are involved in the initial stage of digestion, as indicated by a positive reaction for nonspecific esterase activity along the entire intestinal lumen.

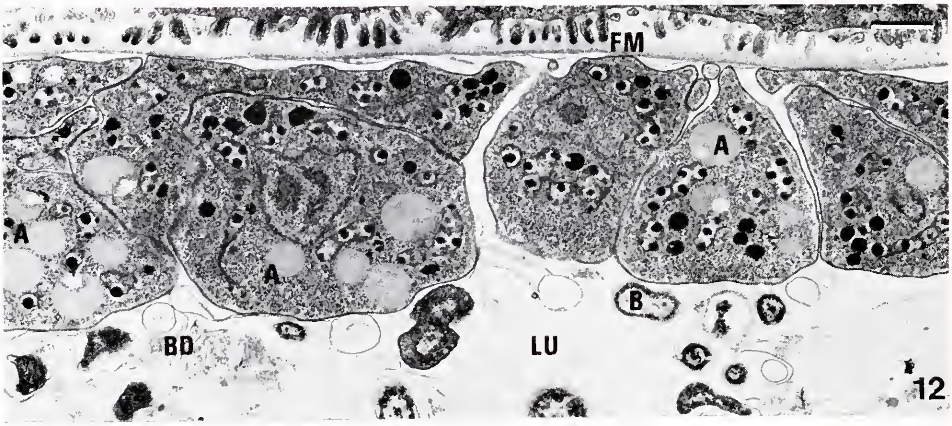
The esterases and other enzymes involved in luminal digestion may possibly be secreted by the esophageal gland cells, the valve cells, and the anterior secretory cells of the intestine. Luminal digestion may be the rate-limiting step in the digestive process, because well-fed nematodes subsequently deprived of food for four days still had intestines full of undigested food cells. This might indicate low concentrations of enzymes in the lumen.

At the same time that food enters the intestinal lumen, the intestinal cells, possibly the anterior secretory cells, secrete material for the fibrillar matrix. This matrix becomes sparse in starving animals, in contrast with well-fed specimens, which have a very dense matrix with homogeneously finely fibrillar material.

Well-fed specimens also had vesicles sprinkled throughout the anterior fibrillar matrix and along the length of the intestinal lumen, suggesting that the production of the matrix is coincidental with the ingestion of food.

Once the food has been broken into molecular size in the intestinal lumen, it presumably passes through the fibrillar matrix. This is suggested by the complete absence of detectable food particles in the matrix.

The presence of large numbers of acid phosphatase-positive lysosomes in the intestinal cells indicates that there is an intracellular phase of digestion. This phase probably begins in the less electron-dense cell inclusions and ends in the more electron-dense lysosomes. There are greater numbers of the former type of cell inclusion in well-fed specimens compared to specimens on diets of low-density food: the latter have from 0 to 4 of these inclusions and the former have about 15 to 40 inclusions in transverse sections. Further evidence that these inclusions contain food molecules comes from the fact that specimens raised on bacterial diets had intracellular inclusions filled with homogeneous material (Fig. 3) and those raised on algal diets had inclusions filled with fine grainy material.



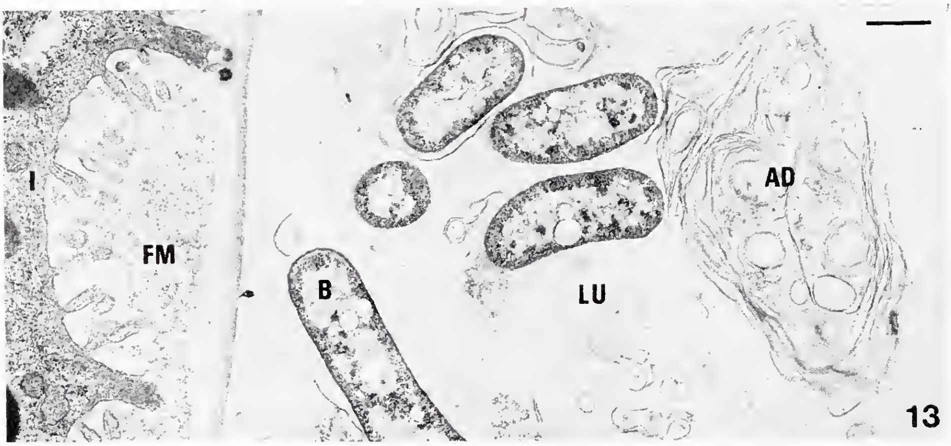


FIGURE 12. Longitudinal section of the intestine of *Diplolaimella* sp. showing amoebae "resting" on the fibrillar matrix. There are undigested and partially digested bacteria nearby. A = amoeba, B = bacterial cell, BD = bacterial cell debris, FM = fibrillar matrix, LU = lumen. Bar is 0.5 μm.

FIGURE 13. Longitudinal section of the intestine of *Diplolaimella* sp. showing the remains of *Dunaliella* sp. and several intact bacteria. AD = algal cell debris, B = bacterial cell; FM = fibrillar matrix, I = intestine, LU = lumen. Bar is 0.5 μm.

Similar grainy material was found in most of the Stage IV inclusions of *C. germanica* and may be characteristic of algal feeding.

Well-fed specimens had large numbers of glycogen particles surrounding both types of cell inclusions, although the lysosomes are usually closer to the luminal border of the cell and thus closer to the edge of the glycogen-containing area of the cell. This suggests that glycogen may be an end product of intracellular digestion. Undigested food debris collects in the posterior portion of the lumen, where it is periodically expelled through the rectum.

A protozoan with pseudopods, an amoeba, was found in the intestinal lumen of *Diplolaimella* sp. (Fig. 12).

Its role in digestion is unknown. Amoebae always occurred in the lumen wherever there was food and were absent from luminal areas devoid of food cells or food debris. In well-fed adults they occurred in numbers estimated at 50 to 100 individuals per worm. The amoebae appeared to be unaffected by the enzymes present in the lumen that breakdown the food cells: no partially digested remains were found in the lumen.

Their morphology indicated that they were similar to other intestinal amoebae and probably actively absorb and digest food.

Digestion in Chromadorina germanica. In contrast to *Diplolaimella* sp., *C. germanica* has a set of three teeth to puncture food cells and ingest their contents. A second set of teeth probably macerate the food further and may prevent particles beyond a certain size from entering the esophagus. Thus, digestion begins with the mechanical breakdown of food in the buccal cavity. Cell envelope fragments were never observed in the gut, so that only cellular contents and not cell envelopes must be sucked down through the esophagus. No enzymatic reactions were detected in the esophageal gland cells, suggesting that the action of these cells in secreting enzyme precursors is similar in both species. The food particles continue to the intestinal lumen. Here, they are further broken down by esterases and possibly by other enzymes. Then food molecules are absorbed by the intestinal cells, presumably by endocytosis. Digestion then proceeds in the various cell inclusions, probably begininning in the Stage I or Stage III inclusions. The final stage of digestion occurs in the Stage IV inclusion. The large amounts of secretory products found in the intestinal cells are primarily used in the lysosomal digestion of food. Digested food is stored as glycogen and, rarely, as lipid.

Results of feeding on different foods

Various species of algae and bacteria were fed to the two species of nematodes including two species of bacteria, four species of chlorophytes, and two species of diatoms. The bacterial cells were smallest, being about 2.2 μm in length. The chlorophytes ranged approximately from 2.2 to 4.9 μm in diameter or length. The diatoms were the largest at more than 10 μm long.

C. germanica survived on diets of the diatoms and three of the chlorophytes: *Dunaliella* sp., *D. salina,* and *Chlorocccum* sp.; but it was unable to survive on *Nanochloris* sp. and the bacterial species tested. *Diplolaimella* sp. was able to survive on the bacteria and chlorophytes but eventually died on diets of the diatoms. Since the cultures of both worms were contaminated with bacteria, starvation was a lingering process for *Diplolaimella* sp. It was probably able to use this alternative food source.

These results were borne out by examination of the guts of specimens fed various diets. The species of bacteria, D5-2 and A5-6, were found in the intestinal lumen of *Diplolaimella* sp. as both intact and partially digested cells (Fig. 11). No matter what else was offered to *Diplolaimella* sp., bacteria were always observed in the intestinal lumen of this animal (Fig. 13). On the other hand, bacteria were never identified in the intestinal lumen of *C. germanica.* Intestinal cells of specimens exposed to bacteria for periods longer than 2 hr wre in various stages of dying.

Undigested and partially digested *Nanochloris* sp. cells were easily identified in the intestinal lumen of *Diplolaimella* sp. Intact or partially digested algal cells were never observed in the gut of *C. germanica.* As in the case of bacterial feeding, the intestinal cells of specimens offered a diet of *Nanochloris* sp. appeared to be undergoing cell death. Remnants of *Chlorococcum* sp., particularly the distinctive starch grains, were found in the intestinal lumina of both nematode species. Broken cell fragments of *Dunaliella salina* occurred in the lumen of *Diplolaimella* sp., whereas both intact cells and cell fragments (Fig. 13) of *Dunaliella* sp. were found in the lumen of this nematode. Remains of neither *Dunaliella* species were found in *C. germanica,* although the intestinal cells appeared normal and were not all dying.

Cell fragments of *Amphora acutioscula,* a diatom, were found in the lumen of *C. germanica.* No cellular debris was found in the lumen of *Diplolaimella* sp. on this diet. Those specimens that did survive for a time on this diet had bacteria in the lumen. No recognizable fragments of the diatom *Cylindrotheca closterium* were found in the lumen of either nematode species, although the intestinal cells of *C. germanica* appeared normal—an indication that the food was being digested.

Discussion

The nematode species studied here have different methods of ingestion and digestion which are intimately related to their respective diets. *Diplolaimella* sp., due to its lack of buccal armature, ingests food solely by the sucking action of the esophagus. Its diet is limited to whole cells somewhat less than 3.9 μm in diameter, which include bacteria and the smaller chlorophytes, and cell fragments from cells greater than 3.9 μm in diameter, which include the thin-walled chlorophytes. This nematode is not anatomically equipped to exert enough pressure on the thicker, more rigid diatom wall to remove diatom cellular contents. The cell size limitation is actually not a direct result of the buccal capsule size, which is only about 1 μm in diameter. More likely, the cell size is a function of the diameter of the temporarily widened buccal capsule and esophagus during ingestion. The *Diplolaimella* sp. digestive tract, with the major portion of digestion occurring in the lumen, is adapted for processing whole bacterial and chlorophyte cells and chlorophyte cell fragments. It is interesting to note, in this regard, that *Monhystera denticulata,* a species closely related to *Diplolaimella* sp., produces β-glucuronidase in the intestine (Jennings and Deutsch, 1975). *Chromadorina germanica* does not produce this enzyme. This enzyme is probably utilized in hydrolyzing the cell envelope of ingested food cells because it is able to attack the glycan or polysaccharide portion of peptidoglycan (Ghuysen, Tripper and Strominger, 1966), a major constitutent of the bacterial cell envelope, and the uronic acid residues composing the mucilages forming many algal cells walls (O'Colla, 1962).

In contrast, the buccal capsule armature of *C. germanica* enables it to ingest algal cell contents. It seems unable to ingest food cells less than 2.2 μm in diameter, probably because the pads and knobs forming the mouth are unable to hold and position these small food cells for piercing by the teeth. Cells less than 2.9 μm, the oral diameter, may either bounce off the teeth during attempts at ingestion or be filtered out by the esophageal teeth, the onchia, during ingestion. *C. germanica* also has a larger esophageal bulb which enables it to exert more suction on the diatom cell contents. Its digestive tract, therefore, is adapted for processing small algal food particles denuded of their cell envelopes.

The function of the nematode esophageal-intestinal valve has generally been thought to be, as its name implies, valvular (*e.g.*, Chitwood and Chitwood, 1950; Bird, 1971). In *Diplolaimella* sp., the circular muscle fibers between the esophagus and valve may function as a valve, but the position of the radiating muscle fibers of the valve itself suggest that this part of the valve, like the esophagus, pumps food through its lumen into the intestine. The protein synthesizing cells of the valve suggest a protein secretory role for these cells, and they may be secreting digestive enzymes involved in the digestive process in the intestinal lumen. This secretory function was also pointed out by de Man (1880) when he described a closely related species, *Monhystera vulgaris*, and observed a "cone-like group of gland cells" between the esophagus and the beginning of the intestine. In *C. germanica,* the valve is less complex and lacks any muscle fibers. Its ultrastructure also suggests that it may be secreting digestive enzymes, as well as acting as a valve structure.

The fibrillar matrix of *Diplolaimella* sp. has not been found in any other nematodes, including free-living and parasitic forms. This matrix may be acting as a molecular sieve, allowing only individual food molecules to pass into its regions. Although the present study did not test this idea, the concept of a molecular sieve is supported by evidence showing that the interface zone of the matrix contains cuticular proteins or cuticular protein-like molecules. If this zone is like a cuticle in chemical composition, then it would certainly limit the size of substances passing through it. The fibrils of the inner matrix zone may function in a type of contact digestion (Ugolev, 1965) in which food molecules may become adsorbed onto the fibrils, where enzymatic activity may then further hydrolyze them. This may be the reason for this matrix zone increasing in density with increased ingestion of food. The matrix may also be similar in function to the periotrophic membrane found in the midgut of certain insects (Smith, 1968) and crustaceans (Sheader and Evans, 1975) where it is believed to protect the intestinal cells from abrasion by hard particulate food. The periotrophic membrane encloses the food being digested and breaks up in the rectum to pass out, as fragments, with the feces (Jennings, 1972). In *Diplolaimella* sp., the fibrillar matrix was never observed to enclose the food, food was never seen embedded in it, nor did it occur with the undigested food debris. Nevertheless, the fibrillar matrix may function, in part, to protect the microvilli from abrasion by the relatively large food particles, in addition to its probable role of molecular sieve and substrate site for contact digestion.

The microvilli found in *Diplolaimella* sp. are different than those occurring in the intestinal cells of other nematodes. The ultrastructure of these microvilli, whereby the electron-transparent material in the microvillus is bounded by a continuation of the endoplasmic reticulum, appears to be unique. This cisterna may be involved in the transport of food molecules from the microvillus to the lysosome, particularly since the microvillus swells in response to high levels of food being digested in the lumen.

Most nematodes have both lipid and glycogen storage inclusions in their intestinal cells (*e.g.*, Bird, 1971). The lack of lipid in *Diplolaimella* sp. and its low amount, relative to glycogen, in *C. germanica* may be an adaptation to short-term storage of food (Calow and Jennings, 1974).

The amoeba found in the *Diplolaimella* sp. intestine is probably ingesting some of the food of this nematode. It is possible that the amoeba is secreting enzymes that may be used by the nematode in luminal digestion or that the amoeba is excreting partially digested food that the nematode then utilizes. Any studies measuring food utilization by *Diplolaimella* sp., such as those for other free-living nematodes (Duncan, Schierner and Klekowski; 1974; Marchant and Nicholas, 1974; Nicholas, Grassia and Viswanathan, 1974), would have to consider that not all the food ingested by the worm is available for digestion.

Although one type of cell is present in the intestine of *C. germanica,* this cell probably goes through different phases of activity at different stages of its life. In a probable scheme, these cells may first be absorptive and digest food within their lysosomes. Older cells then become secretory and package digestive enzymes which are eventually released into the lumen. Then the cell dies. A similar cycle of growth of the intestinal cells described here does occur in a marine nematode, *Pontonema vulgaris,* (Jennings and Colam, 1970) where the cells are first absorptive and then, when they have aged, undergo a period of merocrine secretion.

A major difference between digestion in these two nematodes is in the emphasis on either luminal or lysosomal digestion. *C. germanica* has relatively fewer luminal stages to its digestion of food, as the major part of digestion occurs intracellularly by virtue of the elaborate set of cell inclusions and lysosomes described here. *Diplolaimella* sp. relies much more on luminal digestion, with only the final stages of digestion occurring intracellularly.

There may be an absolute limit on the number of different digestive enzymes one cell is capable of synthesizing. Thus, *C. germanica,* with its emphasis on lysosomal digestion, probably secretes fewer species of enzymes. To optimize its digestion, *C. germanica* must have a fairly narrow diet which would present the intestinal cells with fewer different molecules to hydrolyze, as each food cell has its own unique set of macromolecules. *Diplolaimella* sp., with its emphasis on luminal digestion, may be better adapted to digesting a greater variety of foods. Its few secretory cells may actually be capable of synthesizing a greater number of different enzymes. Its diet, compared to *C. germanica,* is broader, because it feeds on both algae and bacteria.

The phenomenon of selective feeding is shown here to be a function of the size of the food and also, for *Diplolaimella* sp., the type of cell envelope. As a result, these two species have the potential to overlap in their utilization of food resources when chlorophytes of a size utilizable by both are available but do not compete for diatoms and bacteria. Other studies also show that *C. germanica* prefers diatoms and *Diplolaimella* sp. prefers bacteria (Tietjen and Lee, 1977). Selective feeding, then, may be an adaptive method for optimizing digestion—extracting the maximum usable molecules from the ingested food. In this way, a large number of nematode species in a benthic area could be avoiding interspecific competition while exploiting, as a taxon, a large diverse food source.

I wish to thank Drs. John J. Lee, Joseph Osinchak, Patricia Dudley, and J. B. Jennings for their advice and encouragement throughout this work. I especially wish to thank Dr. J. B. Jennings for first interesting me in the study of digestive physiology.

SUMMARY

1. A comparative study has been made of gut ultrastructure, ingestion and digestion of a variety of foods by two salt marsh nematodes: *Chromadorina germanica* and *Diplolaimella* sp.

2. *Diplolaimella* sp. has a small, unarmoured buccal cavity. It has a complex esophageal-intestinal valve that consists of muscle and gland-like cells. The intestinal cells are of two types: the anterior four cells are secretory and the remaining cells are absorptive. There are unusual microvilli lining the luminal borders of these cells that contain a cisterna along the central axis that is continuous with the cisterna of the endoplasmic reticulm. An unusual, structurally complex, fibrillar matrix lines the luminal cell borders. An amoeba is found residing in the intestinal lumen wherever food is present.

3. *Chromadorina germanica* has a larger buccal cavity with two sets of three teeth. The intestine consists of secretory cells that are periodically sloughed off into the intestinal lumen. These cells have a variety of cell inclusions.

4. The process of digestion is described for each species. *Diplolaimella* sp. ingests whole cells or large cell fragments. Digestion begins chemically in the intestinal lumen with secretions from the esophageal gland cells and esophageal-intestinal valve cells. It is completed intracellularly in the intestinal cell pigment granules, which are demonstrated to be lysosomes.

5. *C. germanica* pierces its food and ingests the cell contents. Digestion continues in the intestinal lumen with secretions from the esophageal gland cells and the intestinal cells. Digestion is completed intracellularly in a variety of lysosomes.

6. *Diplolaimella* sp. ingests whole bacteria and small chlorophytes and large cell fragments of the larger chlorophytes. It does not ingest diatoms. *C. germanica* ingests the cell contents of diatoms and large chlorophytes but does not ingest small chlorophytes or bacteria.

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VITELLOGENESIS IN A PRIMITIVE TERMITE, *ZOOTERMOPSIS ANGUSTICOLLIS* (HAGEN) (HODOTERMITIDAE).

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The sources and controls of egg storage proteins, the vitellins, have been subject to increasing attention in the past decade. Vitellogenesis offers an attractive model system in which large amounts of specific proteins are produced in a relatively short period of time, often under the direction of gonadotrophic hormones. In non-mammalian vertebrates a serum precursor to vitellin, a vitellogenin, has been demonstrated to be synthesized in the liver under the direction of estrogen. No other extra-ovarian tissue has been seriously implicated as a major source of egg storage proteins prior to ovulation. Subsequent to ovulation the oviduct donates a number of proteins which surround the egg. However, γ-globulins are specifically taken up into developing avian oocytes in small amounts serving a presumably immunological-protective rather than a nutritive role (Roth, Cutting and Atlas, 1976). While endogenous synthesis of proteins presumably occurs in all oocytes, in non-mammalian vertebrates it does not amount to a large proportion of stored protein (current review, Wallace, 1978).

In the higher invertebrates the source of yolk proteins has not been found to be consistently extra-ovarian. In the octopus, vitellin seems to be primarily an endogenous product of the ovary (O'Dor and Wells, 1973, 1975). In various crustaceans, despite earlier evidence of extraovarian synthesis of vitellogenins, current reports are convincing that the ovary is the major source of vitellin (Lui, Sage and O'Connor, 1974; Lui and O'Connor, 1976). More extensive studies in insects have established the fat body as the major source of vitellin (current reviews: Engelmann, 1978; Hagedorn and Kunkel, 1978; Wyatt and Pan, 1978). However, despite the fat bodies' preeminence as primary suppliers of vitellin, an ovarian source of yolk protein synthesis has been firmly established for the giant silkworm and, more specifically, the source of this endogenous ovarian protein is the follicle cells (Anderson and Telfer, 1969; Bast and Telfer, 1976).

Our knowledge of the repoductive physiology of social insects is still in an infancy stage, primarily due to the small proportion of each colony which is actively reproductive. Where termites stand relative to other insects with regard to the vitellogenic process is still in doubt. Studies with physogastric queens of the termite *Macrotermes subhyalinus* (Rambur) (Termitidae), which are capable of laying up to 40,000 eggs per day, have been interpreted to suggest that the follicle cells of the ovarioles are responsible for the production of vitellogenins (Wyss-Huber and Lüscher, 1975). Despite the tentative nature of this later weak inference, the termite queens' ability to reproductively support the large monogynous societies of the higher termites is nonetheless unsurpassed among the eusocial insects, and indeed, among the Insecta and the unique social structure and development of termites in general, may have resulted in modifications or exaggerations in the production of vitellogenic proteins compared to other nonsocial insects. Our limited knowledge of the structure and especially the physiology of the termite reproductive system (Weesner, 1969) must be extended. By taking advantage of our current understanding of termite evolution (Emerson and Krishna, 1975) we can perhaps approach an understanding of the origins of the remarkable reproductive capacity of the higher termites. In that light, we have undertaken an investigation of the development of the neotenic reproductive of the primitive west coast damp-wood termite *Zootermopsis angusticollis* (Hagen) (Hodotermitidae). Various aspects of vitellogenesis in this species were studied. We report here the isolation and TEAE-cellulose purification of vitellins from eggs of neotenic reproductives and immunological comparison with vitellogenins from adult and neotenic reproductives. By measuring vitellogenin production relative to oocyte maturation, we offer tentative proof of extraovarian synthesis of vitellogenin. Initial molecular weight estimates of the vitellin subunits also characterize these termite vitellins as similar to the extraovarian vitellins of other insects.

MATERIALS AND METHODS

Culturing of animals

Stocks of *Zootermopsis angusticollis* were obtained from the Del Monte Forest, Pacific Grove, California. Colonies were maintained in wood contained in 37.5 × 20.5 × 17.5 cm plastic boxes at ambient room temperature (25° C). Neotenic reproductives (also termed supplementary or secondary reproductives) were obtained in the following manner: larvae were collected from laboratory stock colonies and maintained in isolated groups, without a functional queen or king, in petri dishes containing wet filter paper and wood from the stock colony. These groups were kept in an incubator at 24° C and 24 hr light. Under these conditions competent larvae molted to neotenic (non-winged) secondary reproductives and began laying eggs 25 days (range 22 to 30 days) after the neotenic molt. For a discussion of the termite neotenic reproductive caste see Miller (1969). Adult (winged) primary reproductives were taken from incipient colonies which had been established in the laboratory.

Preparation of crude yolk sample

Eggs were collected daily from colonies headed by neotenic reproductives and stored at 4° C. Crude yolk was prepared by homogenizing eggs over ice in distilled water. An equal volume of 0.4 N NaCl saline (0.4 N NaCl, 0.02 N PO_4, 0.02% sodium azide, pH 7.5) was subsequently added with further homogenization. The homogenate was centrifuged at 14,000 × *g* for 15 min. at 4° C in an International B-20 refrigerated centrifuge and the supernatant, between the floating lipid layer and the pellet, was collected and stored at 4° C. This constituted the crude yolk sample.

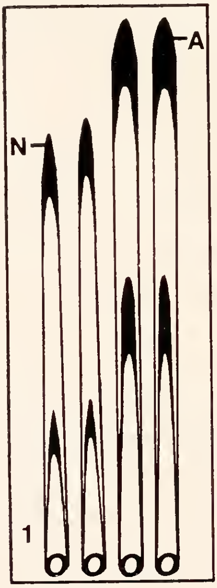
Purification of vitellin from crude yolk

Crude yolk was purified on a TEAE-cellulose (Cellex T, Biorad) column after Pan and Wallace (1974). The column (0.5 × 5.0 cm) was equilibrated with 0.1

N starting buffer (0.1 N NaCl, 0.005 N Na_4EDTA, 0.0125 N Na_2PO_4, pH 6.5) and the crude yolk sample (from 18 mg of eggs) in its, 0.2N NaCl buffered saline diluted 1 : 1 with distilled water, was run onto the column. The sample was eluted from the column with starting buffer in steps of increasing NaCl concentration and, using immunochemical means of identification, the fractions containing the purified yolk (0.3–0.4 N NaCl) were pooled and vacuum dialyzed overnight in the cold against 0.15 N NaCl saline.

Immunological procedures

Antisera were obtained by immunizing two rabbits with purified yolk emulsified (1 : 1) in Freund's complete adjuvant. Blood was taken from the ear vein one week following a booster injection and the γ-globulins were prepared by precipitation with one-third saturated $(NH_4)_2SO_4$. Quantitative (rocket) immunoelectrophoresis was performed on glass slides covered with 0.8% agarose containing antisera following the methods of Axelson, Kroll and Weeke (1973) using a Tris-EDTA-Citrate buffer, pH 8.6. Hemolymph samples from termites were obtained by pricking the abdomen and collecting the blood in 1 μl glass capillary tubes. The blood was mixed with 20 μl 0.15 N NaCl saline and applied to wells (2 mm diameter, capacity approximately 3.6 μl) which had been precut in the agarose.



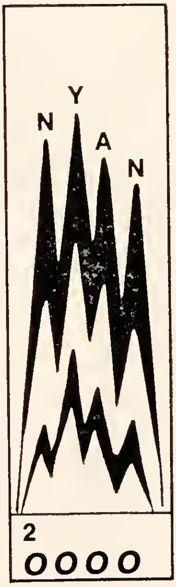


FIGURE 1. Schematic drawing of quantitative "rocket" immunoelectrophoresis of hemolymph samples from female neotenic (N; pair at left) and adult (A; pair at right) reproductives of *Z. angusticollis* on 0.8% agarose containing 3% antibody.

FIGURE 2. Schematic drawing of "fused rocket" immunoelectrophoresis of hemolymph antigens from female neotenic (N) and adult reproductives (A) and purified yolk protein from neotenic reproductives (Y) on 0.8% agarose containing 3% antibody.

Polyacrylamide gel electrophoresis

Partial characterization of TEAE-cellulose purified yolk was carried out by SDS-polyacrylamide gel electrophoresis following the methods of Weber, Pringle, and Osborn (1972). Phosphorylase a, pepsin, trypsin and lysosyme were used as molecular weight standards. Polyacrylamide gel electrophoresis was carried out in 3.5% gels with Tris-EDTA-Citrate buffer, pH 7.5. Gels were stained with Coomassie blue R250 and scanned using a Gilford 240 spectrophotometer.

RESULTS

Identity of hemolymph and yolk antigens

Rocket immunoelectrophoresis of hemolymph samples collected from female neotenic and adult reproductives showed two precipitin peaks (Fig. 1). Hemolymph samples from female larvae and male larvae and from male neotenic and male adult reproductives showed no visible reaction with the antisera to crude yolk by this method. Fused rocket immunoelectrophoresis showed a reaction of identity of the neotenic hemolymph antigens with the TEAE-cellulose purified vitellins from neotenic reproductives and with hemolymph antigens from adult

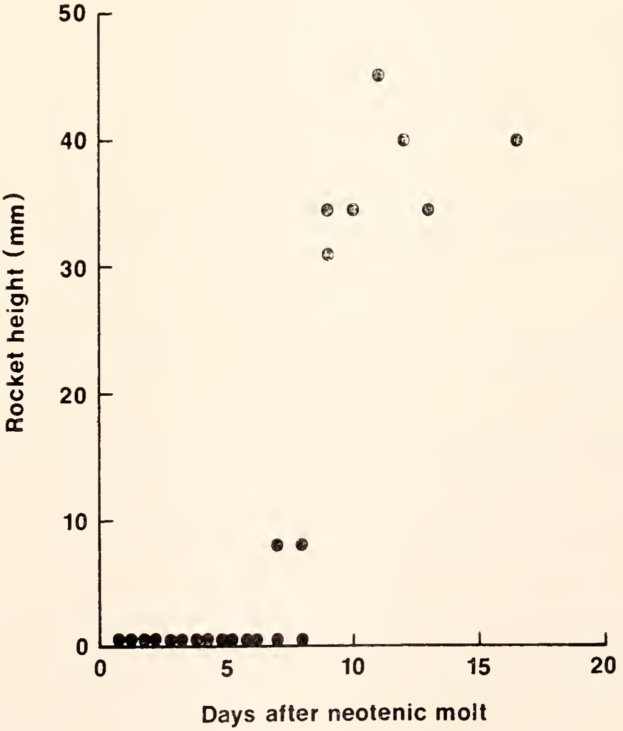


FIGURE 3. Relative concentration of vitellogenin in 1 ul hemolymph samples taken from female neotenic reproductives from day 1 to 18 days after the neotenic molt. Each point represents a sample taken from a different animal.

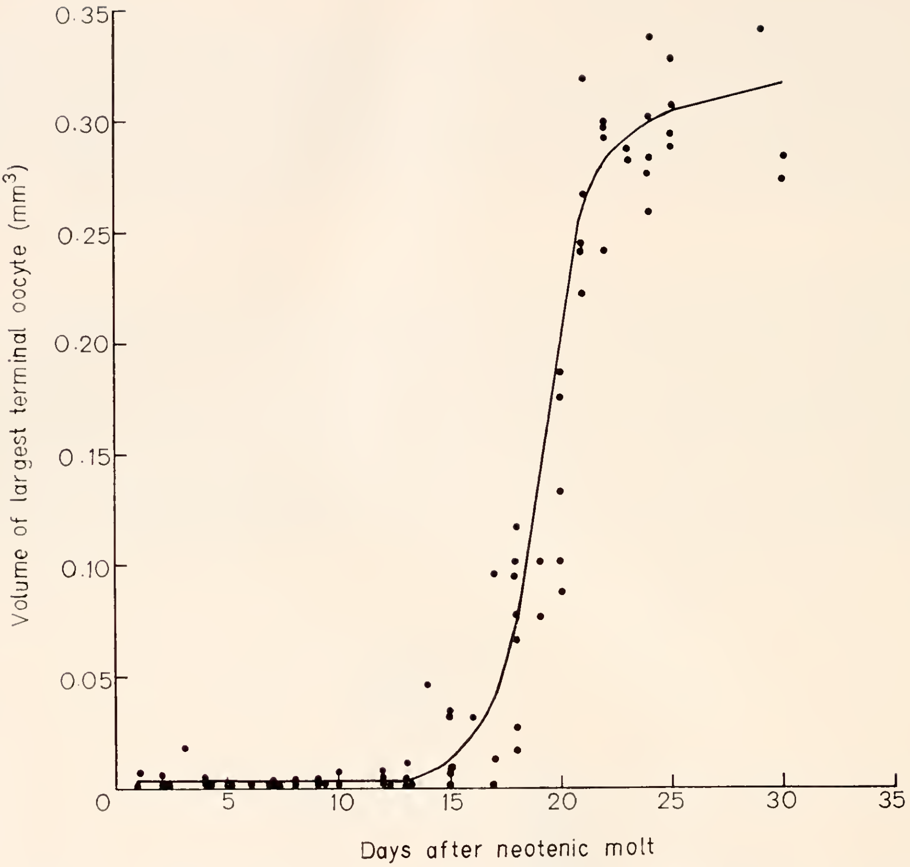


FIGURE 4. Volume (mm^3) of largest terminal oocyte from *Z. angusticollis* neotenic reproductives from day 1 to 30 days after the neotenic molt. Oviposition occurred between days 22 and 30 (mean day 25).

reproductives (Fig. 2), therefore the hemolymph derived antigens are immunologically identical with the vitellins and are aptly called vitellogenins.

Timing of hemolymph vitellogenin titre and oocyte growth

Hemolymph from female neotenic reproductives was not found to contain the vitellogenin until seven to nine days after the neotenic molt (Fig. 3). Ovaries from neotenic reproductives subsequently showed rapid growth between days 15 and 20, resulting in the oviposition of mature eggs between 22 and 30 days (mean 25 days) after the neotenic molt (Fig. 4). Over a period of three weeks, between 55 to 130 eggs were laid, or an average of 2.6 to 6.1 eggs per day. The accumulation of vitellogenin in the hemolymph is probably not derived from atretic follicles, since the vitellogenin appears in the hemolymph prior to any visible yolk deposition.

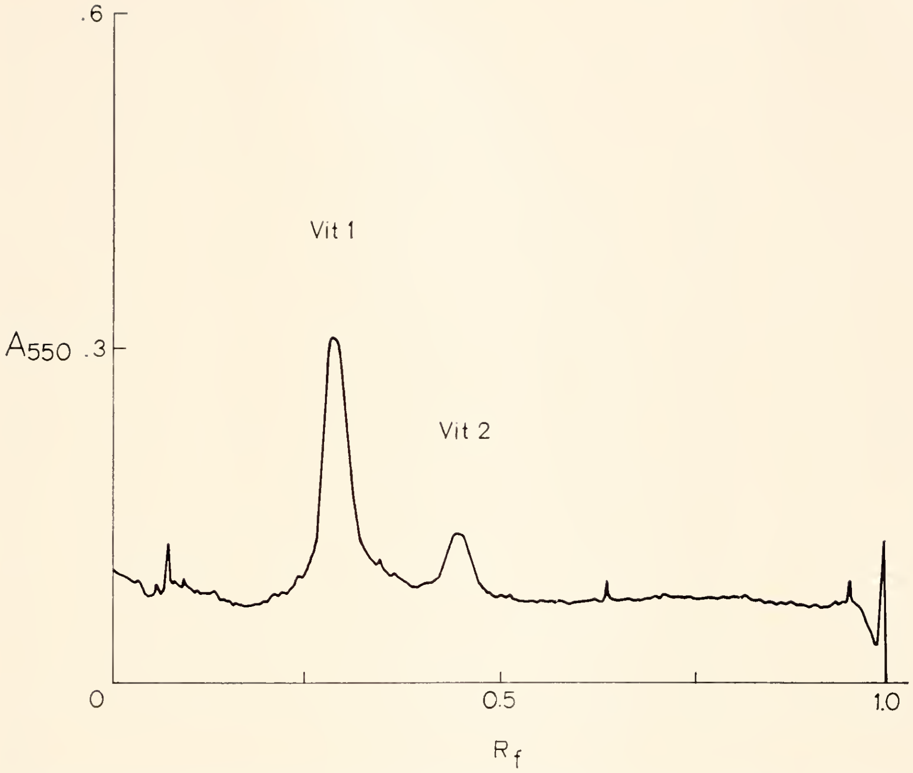


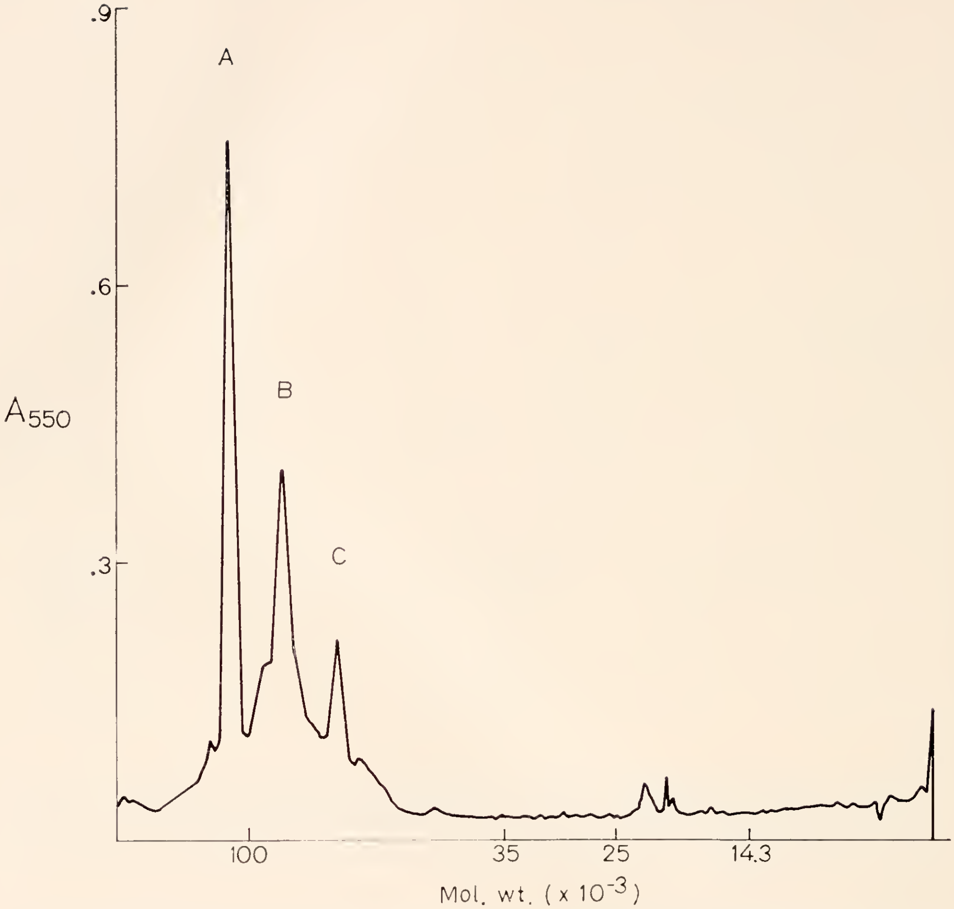
FIGURE 5. Gel profile of purified yolk protein from female neotenic reproductives separated by acrylamide gel electrophoresis (pH 7.5, 3.5% gel, Tris-EDTA-Citrate buffer), and showing the two vitellins (Vit. 1 and Vit. 2), using a Gilford Spectrophotometer (A_{550}). Proteins were stained with Coomassie blue R250.

Properties of termite vitellin

Gel electrophoresis of TEAE-cellulose purified vitellin from day-old neotenic reproductive eggs in nondenaturing conditions gave two distinct bands (Fig. 5), showing the existence of two distinct vitellins. Preliminary characterization of this same sample on SDS-gel electrophoresis gave three major bands (Fig. 6). The molecular weights of the two fastest traveling components were estimated to be 89,000 and 71,000 Daltons, respectively. The slowest traveling component did not travel as fast as phosphorylase a and was estimated by extrapolation at 112,000 Daltons.

DISCUSSION

The major yolk proteins, vitellins, of insects are normally found only in female reproductives and are transported to the ovaries via the hemolymph as vitellogenins. This report represents the first description of the female specific hemolymph proteins of a primitive termite, *Z. angusticollis,* and, indeed, of any of the Isoptera. The hemolymph proteins described in this report are undoubtedly vitellogenins



FIGURES 6. Purified yolk protein from female neotenic reproductives, separated by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide). Molecular weights represent those of the standards phosphorylase a, 100,000; pepsin, 35,000; trypsin, 25,000; and lysosome, 14,300 Daltons. The molecular weights of the three major subunits were estimated to be A, 112,000; B, 89,000 and C, 71,000 Daltons.

since, as in other insects, they were found only in female reproductives, appeared in the hemolymph prior to oocyte growth, and became the major proteins in the mature oocytes. The hemolymph vitellogenins are, furthermore, immunologically identical to TEAE-cellulose purified vitellin obtained from eggs. The hemolymph vitellogenins are also immunologically identical in both the neotenic and adult reproductives of *Z. angusticollis.* Thus, there do not appear to be any specific differences in the vitellogenins produced by the two reproductive sub-castes of this social insect species. This is similar to what has been found for the honey bee *Apis mellifera* where the female specific protein of workers and queens is immunologically identical (Rutz and Lüscher, 1974).

The data demonstrate that in neotenic reproductives of *Z. angusticollis,* vitellogenin is first produced, or at least released into the hemolymph, seven to nine days after the neotenic molt. It is not known what regulates vitellogenesis in this species, but nutrition or other colony interactions may play an indirect role; neotenic reproductives isolated soon after the molt from the parent colony and kept as heterosexual pairs had levels of hemolymph vitellogenin close to zero and even after 60 days had not laid eggs (Greenberg, 1978). These isolated neotenics do not receive the benefits of communal relations with other colony members, such as grooming and food exchange, which may be important for normal reproductive development. The control of ovarian development in neotenic and adult reproductives may not be entirely similar, however, in that, in contrast to neotenics, adult reproductives must leave the colony and become established as isolated pairs for oogenesis to occur normally (Hewitt and Nel, 1969). Ovary development in the adult reproductive appears to be inhibited by tactile stimuli received from other termites in the colony (Hewitt, Watson, Nel and Shoeman, 1972). There is evidence suggesting that ecdysone is ultimately necessary to stimulate oocyte maturation and protein synthesis in the ovary of the termite queen (Lüscher, 1976). Juvenile hormone, which plays a key role in cockroach vitellogenesis (Kunkel, 1973; Engelmann and Friedel, 1974), may not be necessary or may even have an inhibiting effect in termites (Lüscher, 1976). Indeed, preliminary attempts to induce vitellogenesis in *Z. angusticollis* neotenics by injection of juvenile hormone have not been successful (Greenberg, unpublished data). This lack of a role of JH would be surprising, however, considering the close relationship of cockroaches and termites (McKittrick, 1965; Kunkel and Lawler, 1974).

Preliminary characterization of the TEAE-cellulose purified yolk proteins of *Z. angusticollis* suggests that they are similar in solubility properties (being insoluble in low ionic strength media) to vitellin from other insects (Kunkel and Pan, 1976; Gellissen, Wajc, Cohen, Emmerich, Applebaum and Flossdorf, 1976; Engelmann, 1978) and are composed of several subunits. The assignment of the 3 major subunits to the two native vitellins is not yet clear, nor is the subunit composition known for the hemolymph vitellogenin. On the basis of immunological identity, however, it appears that the hemolymph vitellogenins and the egg yolk vitellins are indistinguishable. The presence of two hemolymph vitellogenins in this termite species is not surprising in view of the fact that multiple vitellogenins have been found in the cockroaches *Periplaneta americana* by Bell (1970) and *Brysotria fumigata* by Barth and Bell (1970). No other specific data of this nature is available for comparison, however, for any other of the Isoptera.

The site of synthesis of the termite vitellogenins described in this report is not known, though there is considerable evidence suggesting that in other insects the fat body is the site of synthesis of these proteins (Pan, Bell and Telfer, 1969; Hagedorn and Judson, 1972; Chen, Couble, Delucca, and Wyatt, 1976). Since the major egg yolk proteins described in this study are antigenically identical to proteins found in the hemolymph prior to oocyte growth, we are inclined to believe that vitellogenin production in this primitive termite, *Z. angusticollis,* takes place in the fat body, as in the majority of other insects studied. The data cannot, however, exclude the possibility that part or parts of the vitellin may be synthesized in the follicle cells. This statement is made in view of the fact that Wyss-Huber and Lüscher (1975) have presented evidence which they interpret as suggesting that the ovarian follicle cells are the main site of vitellogenin synthesis in queens of the higher termite *M. subhyalinus.* The queens of the large societies of the higher termites are uniquely physogastric and are unsurpassed among the Insecta in the enormous quantities of eggs they are capable of producing. As a consequence of this, the entire structure of the abdomen and reproductive system of these queens may certainly have been modified in many as yet unknown ways. For instance, the enlarged ovaries of the higher termite queens occupy the major part of the abdomen and greatly distend it. The sternites and tergites become widely separated by the intersegmental membrane, whose tremendous increase in surface area is not yet understood (Weesner, 1969). Queens of the lower termites, however, do not generally become physogastric to the same extent as do those of the higher termites. Furthermore, the rate of egg laying is much lower. Neotenic queens in this study laid an average of between 2.6 and 6.1 eggs per day. Primary queens of the harvester termite *Hodotermes mossambicus* (Hagen) (Hodotermitidae) were reported to oviposit an average of between 1.3 and 7.5 eggs per day (Watson, 1972). It may very well be that in the higher termite queen the extremely rapid rate of egg production which results in the daily oviposition of tens of thousands of eggs is substantially enhanced by a follicle cell product. It should be pointed out, however, and taken note of that the methods of Wyss-Huber and Lüscher were not specific for termite vitellogenin, but simply measured relative rates of synthesis of unknown proteins by fat body and ovary. Their interpretations are therefore, at best, merely suggestive.

Among other eusocial insects of the Hymenoptera, most of the information available on vitellogenesis concerns species of the genus *Apis* (Reviewed by Engels, 1974). Queen honey bees can produce equal to their own weight in eggs daily and thus require correspondingly large amounts of vitellogenic proteins. It appears, however, that in *Apis mellifera* the yolk proteins are of extraovarian origin and that the oocyte itself, or the follicular epithelium, do not synthesize vitellogenins (Engels, 1974). Although the queen fat body is able to synthesize vitellogenin at a rate sufficient to explain its corresponding incorporation into eggs (Engels, 1972; cited in Rutz and Lüscher, 1974), some of the yolk precursor materials may be obtained from the workers (Engels, 1971, 1974; Rutz and Lüscher, 1974). The transfer of yolk precursor materials from worker bees to the queen could provide for the enhanced vitellogenesis needed for the high rate of egg laying which occurs during the active summer period. It is not known, however, if this type of supplementation can take place in termite colonies. The workers of higher termites are not true adult insects, as are those of the Hymenoptera, but are a terminal larval caste. Nevertheless, they do feed the physogastric queen, who is generally unable to feed herself. It is not known however, if workers of the higher termites produce or transfer vitellogenin precursor proteins to the queen.

In view of the potential importance of the results of Wyss-Huber and Lüscher, however, and the fact that in other insects vitellogenins have been found to be primarily of extraovarian origin, more specific studies are certainly needed to determine the site of synthesis of the termite vitellogenins.

We thank the Forestry Department of the Del Monte Properties, Pebble Beach, California for giving permission to collect termites on its property. This study was supported in part by a Sigma Xi Grant-in-Aid of Research and a University Fellowship to S. L. W. G.; by an N. S. F. grant (GB-32536X and BMS 72-01915 A03) to A. M. S.; and by an N. I. H. grant (AI-11269) to J. G. K.

SUMMARY

1. Vitellogenesis was studied in the primitive termite *Zootermopsis angusticollis.* Quantitative (rocket) immunoelectrophoresis was used to demonstrate two immunologically distinct sex-specific hemolymph proteins, vitellogenins, in female reproductives which were immunologically identical in neotenic and adult reproductive serum and in egg yolk from neotenic reproductives.

2. The vitellogenins are not detectable in hemolymph of neotenic reproductives by our methods until seven to nine days after the neotenic molt. A marked increase in oocyte volume was observed beginning six to eight days later and oviposition occurred approximately 25 days (range 22 to 30 days) after the neotenic molt. Presumably, vitellogenin production in this primitive termite occurs outside the ovary, as in the majority of other insects studied. However, the exact site of synthesis of this protein has not yet been demonstrated in the Isoptera.

3. Acrylamide gel electrophoresis at pH 7.5 of TEAE-cellulose purified egg yolk protein also demonstrated the existence of two distinct vitellins. SDS-gel electrophoresis of purified egg yolk protein showed that the two vitellins are composed of at least three subunits whose molecular weights were estimated to be 112,000, 89,000 and 71,000 Daltons.

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UNUSUAL GENETIC VARIATION IN BODY SIZE, DEVELOPMENT TIMES, OIL STORAGE, AND SURVIVORSHIP IN THE MARINE COPEPOD *PSEUDOCALANUS*

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The techniques of quantitative genetics have long been applied to animal breeding and agriculture, but seldom to studies of natural populations, and even more seldom to marine organisms. Yet they are the only techniques that can be used to study inheritance of most traits of interest to ecologists and evolutionary biologists. The best introduction remains the book by Falconer (1960).

A previous study of the heritability of demographic traits in the marine copepod *Eurytemora herdmani*, although evidently the first of its kind, produced few surprises (McLaren, 1976a). It was found that traits of such obvious demographic importance as body size (influencing fecundity), age of maturity, and mortality had low heritabilities at laboratory temperatures experienced in nature. As McLaren (1976a) notes, such low heritabilities are expected for traits involved in determining fitness. The results of the present study are very different and not so easy to understand.

In what follows it will be clear that we may be dealing with a genetically complex population of animals, and possibly with cryptic or sibling species. Therefore, we refer throughout only to the genus name, *Pseudocalanus*. The genus is among the most widespread and numerically abundant metazoans in the world (Corkett and McLaren, 1978).

MATERIALS AND METHODS

Three experiments are reported in this study. In the first experiment fertile females were selected for extreme sizes from a sample taken near Halifax, Nova Scotia, in February, 1975. Offspring were kept at 10° C in individual 25 ml vials and fed on a variable schedule with the flagellate *Isochrysis galbana*. No attempt was made to keep track of precise development times, but these were certainly retarded beyond those predicted for conditions of food satiation (McLaren, 1974). Seventeen female and four male offspring reached maturity. The cephalothorax lengths of female offspring were measured for comparison with sizes of their female parents (dams). The resemblance between offspring and dams was analyzed by regression to estimate heritability of size.

The second, more extensive experiment was carried out with parents from a sample taken in mid-May, 1975. Experimental designs, rearing methods, and biometrical techniques were largely the same as those used previously for another copepod by McLaren (1976a) and are outlined briefly here. Seventy-five adult males were placed in bottles, each with about 5 immature females in copepodid stage V (hereafter C-V). Neither males nor females were consciously selected or combined with respect to body size or any other trait. Excess food allowed most

TABLE I

ANOVA *and heritability estimates of traits of female offspring of* Pseudocalanus *reared in the laboratory at 10°.*

Trait	Source of variance	Mean square	d.f.	Proportion of total variance	*P* of *F***	h²**
Body size	Between sires	34.61	8	0.230	0.02	0.92
	Between dams, within sires	5.77 (5.48)*	12 (6.6)	−0.021	0.60	0
	Within families	6.80	112	0.790	—	—
Age of maturity	Between sires	77.97	8	0.140	0.13	0.56
	Between dams, within sires	31.63 (35.90)	12 (9.9)	0.148	0.02	0.59
	Within families	14.32	115	0.712	—	—
Time, N-I to C-I	Between sires	21.39	8	−0.110	0.82	0
	Between dams, within sires	34.55 (40.96)	12 (10.8)	0.367	<0.001	1.47
	Within families	8.96	114	0.743	—	—
Time, C-I to adult	Between sires	30.53	8	0.252	<0.001	—
	Between dams, within sires	3.62 (3.06)	12 (5.4)	−0.053	—	—
	Within families	5.89	114	0.801	—	—
Relative size of oil sac	Between sires	13.98	8	0.193	<0.001	—
	Between dams, within sires	2.18 (1.66)	12 (3.9)	−0.064	—	—
	Within families	3.73	115	0.871	—	—

* Terms in parentheses are for reduced d.f., due to unequal class sizes.

** These are based on pooled sums of squares where intermediate level mean squares are non-significant, using the criteria in Sokal and Rohlf (1969); see text.

females to mature, but only a portion of these were successfully fertilized. Females were inseminated only shortly after maturing, and those carrying fertile egg sacs were isolated thereafter. Cephalothorax lengths of parents were measured. The experimental design (see below) called for the rearing of families produced by more than one dam mated to the same male (sire). The families of all such dams (apart from a few whose dams were lost or killed during handling) were reared. A few additional families of single dams mated to individual sires were also reared for regression analyses. The numbers of successful matings and rearings will be summarized in the Results. An attempt was made to obtain a minimum of 18 nauplii (usually two successive clutches) from each dam, but this was not always possible. The experiment was begun with large numbers of stage I nauplii (hereafter N-I) hatched between May 31 and June 7 at times known to within a few hours. Rearing was highly labor-intensive. Each N-I was placed in an individual 25 ml vial, labelled with coded numbers, and placed and treated randomly in a 12L:12D light cycle at 10° C. Food for the offspring consisted of 4-day-old cultures of *I. galbana,* diluted to about 3×10^5 cells per ml with sterilized, enriched sea water to form a culture medium. Every fourth day the animal was removed from its vial by eyedropper and its culture medium changed. Vials with young animals were checked daily so that time to molting into copepodid stage I (hereafter C-I) was established to within about 0.5 day. The animals were checked more frequently thereafter and age of maturity determined to within a few hours. After maturity, animals were removed from their vials and their cephalothorax lengths measured. An outline drawing of the amount of oil (probably largely wax esters, forming a discrete globule, often called an oil sac) was made on a standardized outline of a cephalothorax length; thus, the relative amount of oil, independent of body size, could be ascertained.

The individuals reared in the laboratory in this experiment were either members

TABLE II

ANOVA *for binomial data of survivorship to maturity of* Pseudocalanus, *with estimates of* h^2 *made after probit transformation (see text).*

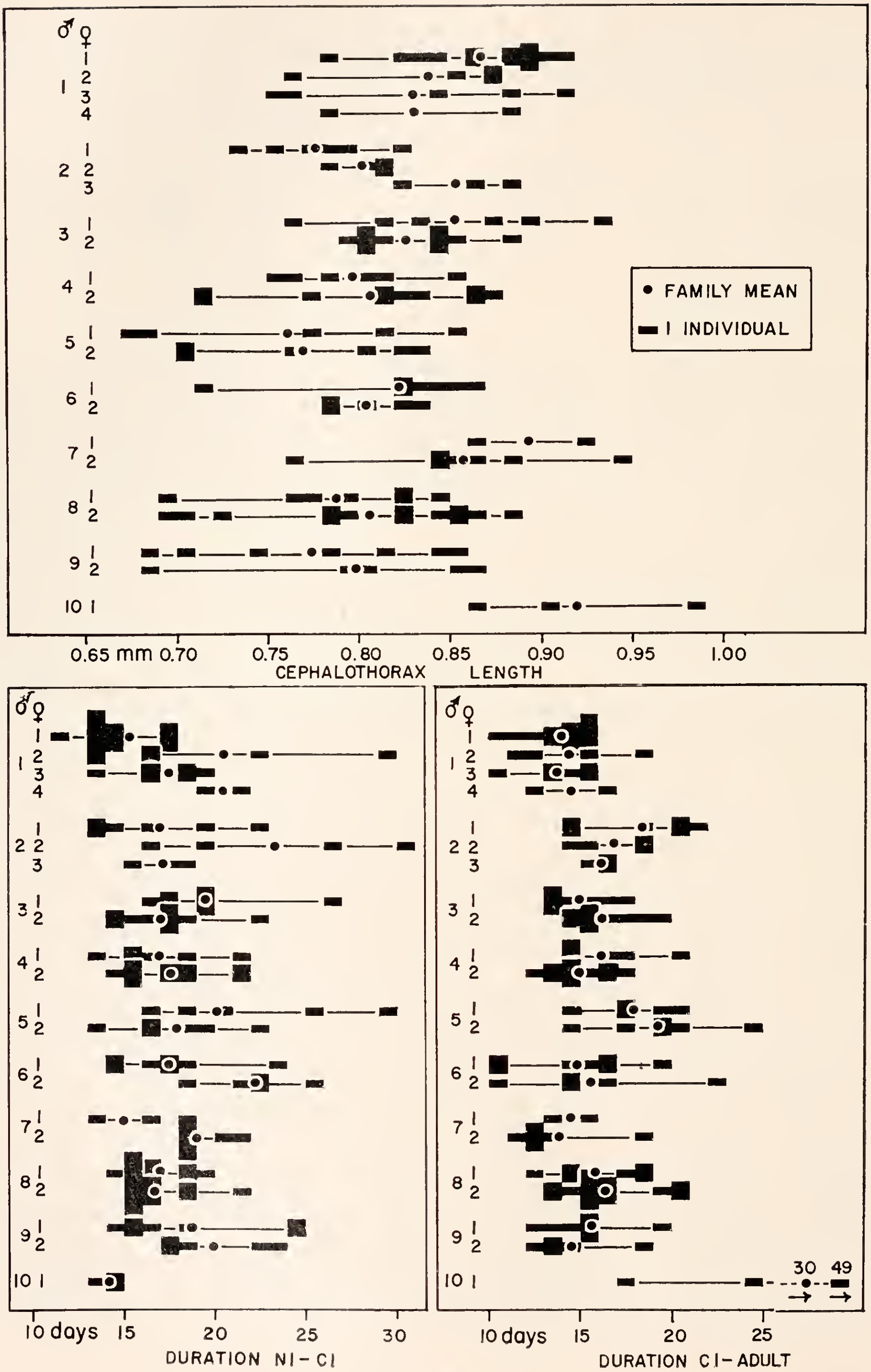
Source of variance	Mean square	d.f.	Proportion of total variance	*P* of *F*	h^2
Between sires	0.340	8	−0.022	0.74	−0.08*
Between dams, within sires	0.539	13	0.076	0.005	0.21
Within families	0.227	347	0.946	—	—

* To be taken as zero.

of the same family (full sibs) or members of families with the same sire but different dams (half sibs). The phenotypic values for six traits (Tables I and II) were analyzed by nested ANOVA, which partitions the phenotypic variance into three components: first, between sires; secondly, between dams within sires; and thirdly, within families (among full sibs from individual dams). These *observational components* should be distinguished from the *causal components,* such as additive genetics variance. Tables I and II do not give direct values for the observational components, but rather express them as proportions of the total variance (*i.e.,* intraclass correlation coefficients). In estimating heritabilities (h^2) of traits from such analyses, the intraclass correlation coefficients between dams within sires are normally multiplied by 4. However, on Table I, where intermediate-level mean squares were non-significant, sums of squares have been pooled, and F ratios and proportions of total variances are recalculated using the decision rules outlined by Sokal and Rohlf (1969). Thus the tabulated proportions of total variance do not always agree with tabulated h^2 values. Survivorship in this second experiment (Table II) is a "threshold" trait (*i.e.,* not continuous like the traits on Table I) for which variance components can be estimated by ANOVA for binomial data (Lush, Lamoreux and Hazel, 1948). Heritabilities on the observed probability scale can then be calculated after probit transformation of variance proportions (Dempster and Lerner, 1950, and references therein).

As will be shown in the discussion, there are statistical and genetic reasons for doubting that these values for h^2 in Tables I and II are true intrapopulation estimates. However, the significance of the tabulated F ratios of the underlying mean squares attest to the reality of the various mean differences that will be discussed.

The third experiment was to look at effects of oil storage on the development of late copepodid stages. Samples were taken near Halifax in mid-to-late August, 1976. From these, C-IV (and some C-V of larger animals, which were scarce as as C-IV) were placed in 125 ml bottles on a revolving wheel (1 rpm) at 10° C and fed periodically with *I. galbana* and the diatom *Thalassiosira* sp. Animals were chosen to give a wide range of sizes of bodies and oil sacs. Amounts of oil in C-V stages as percentages of body volumes were estimated from sketches, using scaled-up models made of modelling clay and determining displacement volumes. Individuals were observed periodically to estimate times spent in C-V (some did not mature before the termination of the experiment).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10 days 15 20 25
30 49
DURATION CI – ADULT

RESULTS

Mating and rearing success in the second experiment

Of the 75 males that were offered females, only 23 were successful: 12 mated with 1 dam, 4 with 2, 3 with 3, 1 with 4, and 1 with 5, giving a total of 38 fertilized dams. One successful mating occurred between a very large male (1.02 mm) and a large female (1.09 mm). Other sires whose offspring were reared averaged 0.80 mm (range 0.77–0.84 mm, s.d. 0.022 mm) and their dams averaged 0.97 mm (0.90–1.03 mm, s.d. 0.037 mm). With the inclusion of the large pair, there was evidence of positive size-assortative mating (regression $F_{1,11} = 5.02$, $P \sim 0.05$); without it, there was none ($F_{1,10} = 0.23$, n.s.). In view of the distinct gap in size between the large pair and other parents, subsequent regressions are calculated both with and without the large pair and their offspring (as a single full-sib family, these offspring could not be used in the ANOVA). This family also had some special characteristics that will be referred to later.

Some of the families of the 38 fertilized females were not used because of accidental loss of adults or shortages of young, and others were discarded because they were single full-sib families, unusable in ANOVA. In all, of 441 nauplii of 27 families of 13 sires at the start of the second experiment, 171 belonging to 26 families of the 13 sires were reared to adulthood. Only 14 of these were males, so that (except in survivorship) only female offspring were sufficiently abundant for statistical analyses. Of these, 147 individuals belonged to 21 families produced by 9 sires that had more than one dam. These half-sib and full-sib families could be used in the ANOVA (another family that died out was used in survivorship analysis), and are pictorially summarized on Figure 1 (together with the single full-sib family referred to above). The remaining 5 full-sib families could be used in regression analyses.

Body size

The first experiment used fertile females collected from nature and selected to represent size extremes, and sizes of the 17 female offspring successfully reared (only 4 males were reared, and were discarded) were strongly related to those of their dams (Fig. 2). The weighted regression coefficient (Falconer, 1963) of 0.49 is normally doubled to give an estimate of $h^2 = 0.98$. The selection of parents as such should give negligible bias in estimating h^2 from regression (Hill, 1970). However, in view of the fact that parents were reared in a different (and variable) environment, and because of evidence (below) that parents of such extreme size may not represent interbreeding genotypes, this high estimate of h^2 must be viewed with caution.

In the second experiment, unlike the first, there was no significant regression (at $P < 0.05$) of sizes of female sibs on sizes of their parents. Weighted regressions (as for Fig. 2) of cephalothorax lengths gave the following: $F_{1,11} = 3.60$ for mean offspring on all sires, and $F_{1,10} = 0.04$ when the large sire (10 on Fig. 1)

FIGURE 1. Distribution of three traits among female offspring of 21 families used in the ANOVA of sib traits. Sires and their dams are numbered. Offspring of the single family of sire 10 are also included as an extreme case, discussed in the text.

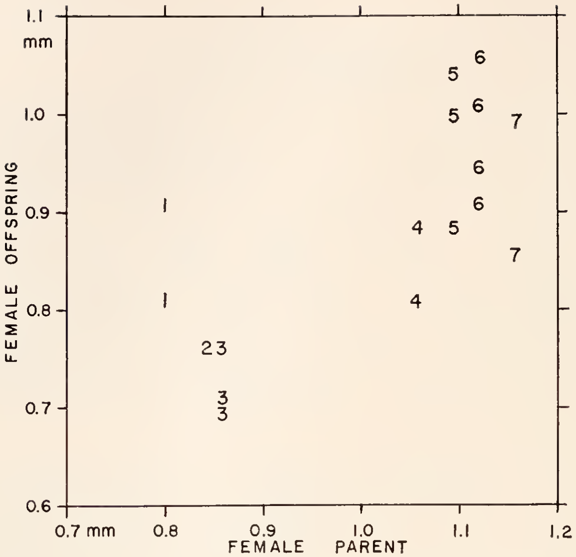

FIGURE 2. Offspring-parent regression. The seven females whose young were successfully reared were chosen from among those of near-extreme size in the sample from nature. Individual offspring are designated by a number indicating the family to which they belong. The weighted regression coefficient (see text) is $b \pm \text{s.e.}_b = 0.49 \pm 0.155$; $F_{1,5} = 7.02$, $P \sim 0.005$.

and his family are excluded; $F_{1,22} = 2.19$ for mean offspring on all dams, and $F_{1,21} = 0.31$ when the dam of the large sire and her family are excluded. It is important to note that the wild-caught parents in this experiment exhibited much narrower size ranges (see above) than did those used in the first experiment (cf. Fig. 2).

Unlike regression analysis, ANOVA of sib traits is designed to estimate heritabilities in a controlled environment independently of phenotypic variance of the parents. The cephalothorax lengths analyzed by the ANOVA are shown on Figure 1, where a strong pattern is evident: half-sib families tend to have very similar mean lengths. The ANOVA (Table I) quantifies this pattern, revealing that the proportion of variance between sires was very high, whereas that between dams within sires was negligible.

Development time

Time taken to reach maturity showed a very different pattern from that for size. The proportions of variance between sires and between dams within sires are about equal, the sire contribution not quite significant by F-test because of lower d.f. (age of maturity, between sires on Table I). However, durations of the period up to appearance of C-I and the period between C-I and maturity appeared to be under very different control. This can be seen on Figure 1, where means for half-sib families differ considerably for duration of N-I to C-I, but show marked similarity for C-I to adulthood. The ANOVA expresses this as a negligible proportion of variance between sires and a very high proportion between dams within sires during the early part of life, and quite the reverse in the later part of life (Table I). Combined, these two effects lead to roughly equal proportions of variance contributed by dam and sire for the whole developmental period (age of maturity, Table I).

The expected temperature dependent durations in adequate food for the durations from N-I to C-I and from C-I to adult female are about 14 and 16 days respectively (McLaren, 1974; Corkett and McLaren, 1978). As indicated on Figure 1, earlier stages were on average retarded (mean 17.9 days for families used in the ANOVA), but later stages were not (mean 15.6 days).

One offspring of the aforementioned large pair (sire 10 and his mate on Fig. 1) behaved anomalously. It spent 49 days between C-I and adulthood, largely because this offspring was suspended in C-V for some weeks. Its two sibs showed no such pattern.

Survivorship

Overall survivorship in the rearing experiment was 39% and was significantly influenced by differences between dams within sires, but not by sires (Table II). Most animals died in early stages and many spent excessively long times as nauplii before succumbing. Even those that survived tended to be retarded in early, but not later stages (see *Development time* above). Because of the way records were kept, it is not possible to test for differences in survival between early and late

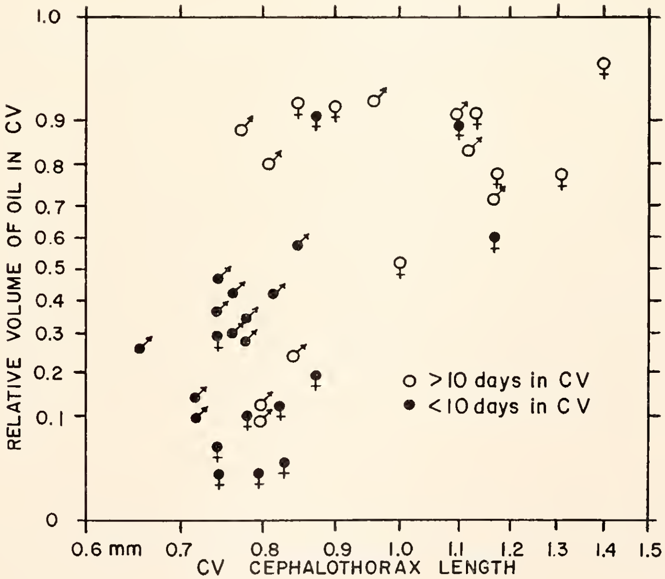

FIGURE 3. Relationships among body size, relative size of oil sac, and suspension of development in a resting stage V copepodid (C V). Relative volume of oil measured as described in the text. Percentage scale transformed by arcsine $\sqrt{\%}$ and cephalothorax length by logarithm, to linearize the relationship.

TABLE III

Spearman rank correlations r_s *of body size with development times and relative amounts of oil. Medians of traits in 21 families (with* $n > 2$*) used in the correlations.*

Test	r_s	P
Time N-I to C-I vs. cephalothorax length	−0.21	n.s.
Time C-I to adult vs. cephalothorax length	−0.59	0.01
Relative size of oil sac vs. cephalothorax length	0.65	0.01

stages, but it appears from indirect evidence that most mortality and therefore the strong between-dam, within sire effect, is early in life.

Oil storage

The ANOVA of relative size of the oil sac among offspring in the second experiment showed the same pattern as that for size and for duration of development from C-I to adult. The proportion of variance between sires was very large, whereas that between dams, within sires was negligible (Table I).

The animals secured from samples from nature for the third experiment were chosen for a wide size range, and some small individuals with a large amount of oil (*against* the trend indicated on Fig. 3) were also selected. Thus a formal regression analysis would not be legitimate, but it is obvious (Fig. 3) that larger animals in general had relatively more oil and that most animals that had large amounts of oil also spent long times suspended in C-V.

Again, the large pair (sire 10 and his dam, Fig. 1) produced unusual offspring. Two contained relatively more oil than any other animals reared, and the third had relatively more oil than all but 4 of 158 offspring in which the oil was measured.

Correlations among traits in offspring

Among offspring reared in the second experiment there was marked negative correlation of body size with duration of C-I to adult and positive correlation with size of oil sac (Table III). This analysis excluded the offspring of the large pair (sire 10 and his dam, Fig. 1), which were anomalous in mean duration of C-I to adult (see *Development time,* above). The individual of this family with the largest amount of oil of any of those reared was also the one that entered a prolonged resting stage in C-V, paralleling the trend described for animals from nature (see *Oil storage* above, and Fig. 3).

DISCUSSION

The results pose two main problems for interpretation. First, the regressions of sizes of offspring on sizes of parents used in the second experiment were not significant, whereas the ANOVA revealed strong resemblances in size among offspring of the same half-sib family. Secondly, the ANOVA showed significant between-sire contributions to variance of offspring body size ($P < 0.05$), time between C-I and adult ($P < 0.001$), and size of oil sac ($P < 0.001$), yet the between-dam, within-sire contributions to these same traits were extremely low and non-significant.

(The very large between-dam, within-sire components in duration of N-I to C-I and survivorship is less problematic, and will be considered below).

The first problem is readily explained by the fact that the parents used in the regression analyses were caught in nature, where much of the variation in their size was probably environmentally induced. McLaren (1963, 1965) inferred from seasonal samples that temperature was the main cause of size variation, and Lock and McLaren (1970) showed that animals reared from C-III to adult female at 5° C averaged 10 to 12% longer than those reared at 12° C. This variation is not much exceeded by the difference between the smallest (0.90 mm) and largest (1.02 mm) dam producing offspring for the ANOVA. As already noted, this phenotypic variance of parents did not affect the ANOVA of sib traits. On the other hand, the largest dam producing offspring in the "first" experiment was fully 45% longer than the smallest. Because of this large difference there was some *a priori* expectation of genetic differences among the smallest and largest families. Indeed, the very high h^2 based on such small samples (see Results and Fig. 2) could itself be interpreted as meaning that the extreme dams chosen represented two species, although they did not differ in any obvious way except in size. It was partly because of these preliminary results that no choice of parents was made in the second experiment.

The second problem poses difficulties in terms of the *casual components* of variance among offspring in the ANOVA. The between-sire proportion of variance is an estimate of the additive genetic variance (*i.e.*, h^2 in the strict sense) of the traits on Table I, whereas the between-dam, within-sire proportion includes non-additive genetic variance (*e.g.*, dominance effects), and maternal and common environment effects as well. Thus, normally in such ANOVA of sib traits the between-dam, within sire proportion of variance if anything exceeds the between-sire proportion (*e.g.*, in the copepod *Eurytemora herdmani;* McLaren, 1976a).

It is very unlikely that the sires could, in fact, have contributed exclusively to the additive genetic variance of such disparate quantitative traits. Although the highly distorted sex ratio (which has occurred in some, but not all experiments with *Pseudocalanus;* see Corkett and McLaren, 1978) is unusual, no obvious mechanism would link it to inheritance through the sire alone.

The only mechanism that appears to account for the results in the ANOVA is assortative mating among parents with respect to *genotype* for body size and correlated traits. In the nested ANOVA design, if sires have mated with dams close to their own genotype for a quantitative trait, then the additive genetic variance contributed by dams will be masked. The rather low mating success, also found by Hart and McLaren (1978) for *Pseudocalanus,* but not by McLaren (1976a) for *Eurytemora herdmani,* suggests that in general mate choice was problematical. Observations of mating "dances" and probable pheromones in *Pseudocalanus* by Griffiths and Frost (1976) may also signify a certain fastidiousness in mate choice. Hart and McLaren (1978) found size-assortative mating by phenotype over a wider range of cephalothorax lengths (males 0.66–0.98 mm, females 0.85–1.03 mm), but this could only be shown in the present experiment with the inclusion of the unusually large pair (sire 10 and his dam, Fig. 1). Among parents producing young for the ANOVA, size as such could not have been used as a phenotypic mating cue by genetically varied mates.

A simple explanation of our results would be that two or more cryptic or sibling species were involved among potential parents, and that genetic differences in size and correlated traits only became phenotypically manifest among their offspring reared in controlled conditions in the laboratory. There is haphazard evidence for the occurrence of "species" of *Pseudocalanus*, differing in size and showing overlapping geographical ranges (see below). However, an *a posteriori* analyses (SNK tests; Sokal and Rohlf, 1969) confirm what seems to be obvious on Figure 1: there is no grouping (at $P < 0.05$) of means of traits of half-sib families into two or more classes that might represent species.

Thus the evidence indicates that the population represented in the ANOVA showed "continuous" variation in genotype for the traits that were measured, and somehow responded to this variation by assortative mating. Although this explains the very high between-sire proportions of variance, it also throws doubt on the actual estimates of h^2 on Table I and II; the statistical and genetical theories underlying such calculations assume random matings of sires and dams.

A possible genetic mechanism underlying size differences in *Pseudocalanus* has already been posed. McLaren, Woods and Shea, (1966) discovered that a large and small form coexisting in a landlocked arctic fiord in Canada had very different DNA content in embryonic nuclei. Woods (1969) concluded that an increase in DNA content of nuclei of this large form probably increased cell size, which in turn would increase body size, if cell number is determinate. Among adult females from Halifax, body size is also strongly correlated with amount of DNA in somatic nuclei (McLaren, 1976b). These curious findings are being explored for publication elsewhere, but clearly may provide a basis for high heritabilities (whether "within" or "among" populations) of body size in the rearing experiment.

Large between-dam, within sire proportions of variance for duration of early stages (N-I to C-I on Table I) and survivorship (Table II) have also been found in traits of the copepod *Eurytemora herdmani* (McLaren, 1976a). Such a pattern is well known in quantitative traits, and the important casual components of such high variance are usually maternal and common-environment effects. It is interesting that in the ANOVA, the observational component of variance was high only in early development and survivorship. Nauplii of *Pseudocalanus* are able to develop up to N-III without food (Corkett and McLaren, 1978). Unhealthy or inadequately nourished dams may have produced young that were less able to develop and survive through critical periods in early life, suggesting a mechanism through which maternal and common-environment effects may operate to produce high between-dam, within sire variance.

The traits examined have demographic significance. Corkett and McLaren (1978) showed that *Pseudocalanus* in temperate waters may undergo several generations of temperature-dependent lengths, uninhibited by evident food shortages. This means that heritable variance in age of maturity and adult female body size (which determines clutch size; McLaren, 1965) could be expressed in nature, as in the laboratory. The negative correlation between body size and development time (Table III) would seem to offer scope for directional selection of shorter generations and larger clutches combined, since both might be advantageous (cf. McLaren, 1974). However, this negative correlation may break down among wider size ranges: Hart and McLaren (1978) showed that embryonic duration of

Pseudocalanus from Halifax was positively correlated with egg size and body size (including a significant partial correlation with size of sire) within the size range over which they demonstrated assortative mating (see above).

Variations in size of oil sac are also probably involved in life-cycle tactics. The oil is clearly an energy store, and is laid down when food is above the level required for maximal rates of growth and reproduction (Corkett and McLaren, 1969, 1978). Most C-V with large amounts of oil among animals collected from nature were clearly in a state of suspended development and most of these were large animals (Fig. 3). These large animals, perhaps also represented by sire 10, his dam and their offspring (Fig. 1), may represent a "species" with a distinct life cycle. In the deeper waters of the Baie-des-Chaleurs, Gulf of St. Lawrence, Lacroix and Filteau (1971) found large C-V animals (mean lengths 1.2–1.3 mm; cf. our Fig. 3) evidently suspended in this stage during summer. They suggested that these were the "form" *major* (= *Pseudocalanus major* G. O. Sars) and that they had an annual life cycle, unlike the smaller "form" which reproduced through the summer.

Probably amount of oil in the smaller animals represented in the ANOVA (Table I) becomes important when they begin to enter overwintering stages. Overwintering stages are known to accumulate as resting C-V before the end of the season of high productivity in some temperate localities (review in Corkett and McLaren, 1978). The present results suggest that larger individuals, whether produced environmentally (temperature; see above) or genetically, would tend to enter a resting stage more readily. These larger individuals would not only be metabolically more secure for a long resting phase than would be the smaller, oil-poor individuals, but would also produce larger clutches upon maturing at the end of winter. A better life-history tactic for genetically smaller individuals might be to forego resting and rather to mature and produce offspring, some of which might be induced by falling temperatures to reach a large size, store more oil, and then enter a resting stage later in the season.

In conclusion, we believe that there is a large amount of genetic variation in *Pseudocalanus* in the waters near Halifax, Nova Scotia. The traits measured are demographically important and possibly subject to strong selective pressures. Although the traits may vary "continuously," at least within certain ranges, the possibility of assortative mating implies that we are not dealing with a single, panmictic population. If, nevertheless, there is enough gene flow among animals represented in our ANOVA so that they might be considered to be a single species, this raises questions about the maintenance of such variation in nature. If the results are interpreted as indicating a complex of species, however difficult to define, then this raises questions about the coexistence of such species in nature. Whatever the true situation, it may also pose problems for conventional systematic analysis and nomenclature.

The work reported here was funded by the National Research Council of Canada. We are grateful to Wayne Fairfull and L. E. Haley for genetic and statistical comments, and to V. Glines, G. Krebs, and S. McCormack for laboratory assistance.

SUMMARY

Body size, development times of copepodid stages, and relative amounts of oil were measured among offspring of *Pseudocalanus* reared in excess food at 10° C in the laboratory. ANOVA of these traits among full-sib and half-sib families showed high proportions of variance contributed between sires (*i.e.,* high heritabilities as normally interpreted), but negligible proportions between dams within sires. Although their parents (of a narrow size range) did not mate assortatively by size, as do those of a wider size range, it is concluded that the suppression of the contribution between dams, within sires, was due to assortative mating, using unknown phenotypic clues, by animals whose genetic differences in size, etc., were unmasked among offspring reared in controlled conditions. Although animals of wider size differences may be distinct species, no significant groupings of traits into two or more classes were found among the offspring treated by ANOVA. Development times of early stages, as well as survivorship (most deaths probably in early life), had high proportions of variance contributed between dams, within sires, and a negligible proportion between sires. These results suggest that underlying maternal and common-environment effects were expressed only early in life. Larger copepodid V stages from nature had relatively more oil and spent longer times developmentally suspended in this stage in the laboratory. Larger animals, whether their size is environmentally or genetically determined, may be more prone to enter such resting stages for overwintering, while smaller animals may benefit more from maturation and further generations. If the animals of the narrower size range near Halifax represent a single species, there are problems about maintenance of genetic variation of their demographically significant traits in nature. If they are a complex of cryptic species, then there are questions about their coexistence. Either situation poses problems for conventional systematics and nomenclature.

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SPERMATOPHORES OF SIX EASTERN NORTH AMERICAN PYRAMIDELLID GASTROPODS AND THEIR SYSTEMATIC SIGNIFICANCE (WITH THE NEW GENUS *BOONEA*)[1]

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The intrafamilial systematics of the Pyramidellidae, one of the largest families of marine gastropods and noteworthy phylogenetically in combining prosobranch and opisthobranch traits, has so far been based on shell characters exclusively. These ectoparasites lack radulae, so even radular characters are missing. This paper, on one aspect of sexual biology, gives the first results of a study determining the relevance of biological characters in the systematics of the east North American odostomioid pyramidellids.

So far as is known, all the Pyramidellidae are simultaneous hermaphrodites. All the British pyramidellids studied by Fretter and Graham (1949, 1962) and by Fretter (1951) apparently transfer spermatozoa directly from one animal to another with an invaginable penis. Mentioning only penial copulation, Fretter (1953) also published a paper on "the transference of sperm from male to female prosobranch, with reference, also, to the pyramidellids." Fretter and Graham were not concerned with lower category systematics, and it is not possible to determine in which genus and species they observed certain things. Maas (1964) observed something of systematic interest not mentioned earlier: that the penises of five species of European pyramidellids are cuticularized and hooked or denticulate in species-specific ways. According to Brandt (1968, p. 278), a brackish water *"Chrysallida"* in Thailand has a penis carrying "two stalked appendages."

One could conclude from the literature summarized above that penial copulation is a familial trait of pyramidellids, but this is not the case. Höisaeter (1965) discovered in Norway that *"Chrysallida" obtusa* (Brown) [1827] [= *"C." (Parthenina) interstincta* (J. Adams, 1797)] produces spermatophores of a characteristic shape, attached to the shell in a constant position. Höisaeter did not understand how these spermatophores function and he did not determine whether there still is a penis. In 1966, Dr. Alan J. Kohn sent me a photomicrograph (still unpublished) of some spermatophores that had been attached to the shells of an as yet unidentified *"Odostomia"* from near Friday Harbor, Washington. I noted the discovery of spermatophores in three east North American species of *"Odostomia"* (Robertson, 1967), and later briefly commented on their systematic significance (Robertson, 1968).

The present paper describes and illustrates the spermatophores of six east North American odostomioid species and shows how some of them vary in appearance and yet how each definitely is species-specific in structure. There are also three species-specific positions that the spermatophores assume on the animals (compare Figs.

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[1] Woods Hole Oceanographic Institution Contribution No. 4100.

30, 44, and 76). A new genus is named in the systematic section, and the paper concludes with discussion of the implications for supraspecific systematics.

The commonest inshore east North American "*Odostomia*" species (all those treated here) are not congeneric with true *Odostomia* of Europe. For reasons given in the section on Generic Relationships of east North American odostomioids, in the two Tables and in the Discussion, the species studied here are grouped in the genera *Boonea* (new) and *Fargoa* Bartsch, 1955. Spermatophore differences helped lead to these conclusions. No definitive systematic treatment of each species is intended here, but taxonomic notes are included to clarify identities and past suppositions about interspecific relationships.

Neither by dissection nor by histology has it been possible so far to determine whether the genital ducts of the American species are as in the British species studied by Fretter and Graham. There are, however, definite differences between the genitalia of the European and American species (Table I, differences 4 and 5). Only once was an extended penis seen in any of the six species. The observations were made on a *Boonea impressa* from Texas which was about to extrude a spermatophore. A short, wide penial papilla was projecting from beneath the mentum to the right of center, and the white spermatophore could be seen inside in transparency. The animal was disturbed during observation, the penial papilla withdrew, and the spermatophore disappeared into the body posteriorly. Presumably, the spermatophore would otherwise have emerged from a pore at the tip of the penial papilla. Dissection of a *B. bisuturalis* confirmed the presence of a penial papilla (at least in *Boonea*). The papilla was withdrawn and not inverted. Its lumen presumably is where spermatophores are formed. The female pore ("genital aperture" of Fretter and Graham?) is on the neck in *Boonea*, but in *Fargoa* it is on the right side of the foot, posterolateral to the right side of the mentum (Figs. 74–76, fp). Spermatozoa somehow are attracted to or drawn into the female pore.

Materials and Methods

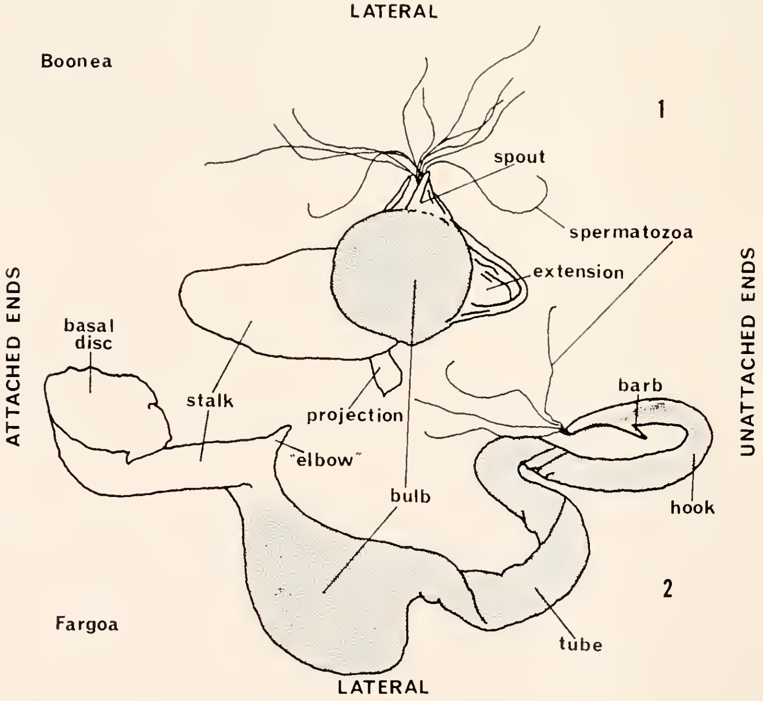
Most of the observations reported in this paper were made at the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, and the Duke University Marine Laboratory, Beaufort, North Carolina. Some data from northwest Florida and Texas are also included. There are some indications of intraspecific geographic variation in the spermatophores of several of the species. The exact localities and hosts are therefore noted for each species.

The spermatophores were obtained preceding, during and (surprisingly) soon after the spawning season. In Massachusetts this seems to be about mid-June to mid-September for the four species there, but in North Carolina this varies among five species and from locality to locality. The dates of spermatophore occurrences and spawning seasons are therefore recorded. Note that most of my work on the living animals was done in the summer months.

The shell-attached spermatophores of *Fargoa* are best located with a dissecting microscope at ×50. The spermatozoa in the freshly produced spermatophores make these a characteristic pale pink. The spermatophores are easily seen on the right sides of the animals. Removal of a fresh spermatophore from a shell is difficult because it is so firmly attached. However, after several days the spermatophores break or automatically loosen and drop off, and they then can be found on the bottom of the container. By then, though, the remaining spermatozoa are heavily infested with bacteria and protozoans. The spermatophores illustrated here without emerging spermatozoa were not fresh.

The spermatophores in the mantle cavities of *Boonea* can be seen when the bodies are extended far out of the shell (Figs. 30, 44), but once seen in this way they are difficult to remove undamaged. To loosen them I put a large sample (preferably 50 or more) of newly collected adult animals in a lidded jar about half full of sea water, and then shook the jar violently. This dislodged some of the spermatophores, and when the water was poured out and swirled in a circular bowl most of the spermatophores settled near the center. They could then be pipetted out individually onto microscope slides.

All drawings of spermatophores in this paper are based on photographs taken with a compound microscope. Even when a spermatophore was obviously bent or folded it is shown here as seen. A problem with the uncuticularized spermatophores is that they are so soft that they are squashed and distorted by cover slips. A feature such as the lateral spout of *Boonea seminuda* shows only if the plane of the squash is right. Series of drawings of randomly selected spermatophores show the real and apparent morphological variation within and between populations of



FIGURES 1–2. Representative spermatophores of *Boonea* (Fig. 1) and *Fargoa* (Fig. 2), showing the terminology used here. No homologies are implied by the orientations and terminological identities. Stippling shows the location of spermatozoa in the bulbs and the tube.

TABLE I

Differences between European and eastern North American odostomioids.

Odostomia, sensu stricto, and "*Chrysallida*" of Europe

1. Protoconchs either with $2\frac{1}{2}+$ tilted heterostrophic whorls (planktotrophic species) or with ca. 1 untilted whorl (lecithotrophic species) [Thorson, 1946, pp. 199–212, figs. 115–124].
2. Operculum with several internal tooth-like processes (Fretter and Graham, 1949, p. 495).
3. Excurrent siphon reduced or lacking (confirmation desirable) [Fretter and Graham, 1949, p. 527].
4. Penial sperm transfer (except in "*Chrysallida*" *interstincta,* which has shell-attached spermatophores); penis invaginable, "whip-like" (Fretter, 1953, p. 220; Höisaeter, 1965).
5. Genital opening on the inner half of the mantle cavity floor, with a ciliated tract running anteriad beneath the right tentacle to the opening of the penial sheath beneath the mentum (Fretter and Graham, 1949, pp. 498–499).
6. Pigmented mantle organ of adult brown-orange, pink (Fretter and Graham, 1949, p. 500), or orange-red (Ankel, 1959, p. 4, fig. 2).

Boonea and *Fargoa* of eastern North America

1. Protoconchs either with ca. $1\frac{1}{2}$ tilted heterostrophic whorls or with ca. 1 untilted whorl (all planktotrophic except Texas *B. impressa*).
2. Operculum without internal processes.
3. Excurrent siphon well-developed (Fig. 30, es).
4. Sperm transferred in spermatophores; penis a short, uninvaginable papilla (*Fargoa*?).
5. Female pore (genital opening?) on neck (*Boonea*) or posterolateral to right side of mentum (*Fargoa*); no ciliated tract.
6. Pigmented mantle organ of adult yellow with a little brown, or dark brick red (*F. bartschi* only).

the three *Boonea* species. All drawings of isolated spermatophores are adjusted to the same scale to facilitate size comparisons.

Terminology adopted here is shown diagrammatically in Figures 1 and 2. Excepting Figure 1, all the illustrations show the attached end to the right in the three *Boonea* species and to the left in the three *Fargoa* species, *i.e.,* as they would be seen from the right *in situ* on the upright animals. "Stalks" refer to a solid structure at the attachment end; "bulbs" and "tubes" (shown stippled) contain spermatozoa.

Measurements, which can be determined from the illustrations, were made of all the spermatophores that were studied: total lengths, maximum bulb diameters, and stalk, tube and extension lengths. Ranges, means, standard deviations and variances were then determined. Most features measured were found to be so variable that further note is taken here only where there appear to be statistical differences.

GENERIC RELATIONSHIPS

The east American species have usually been grouped in the genus *Odostomia* Fleming, 1813, on the basis of shell characters. Table I shows that there are some profound biological differences between the eastern and western Atlantic species, and that they have been wrongly considered congeneric. Alternative generic names for the American species are difficult to find. All the prior subgeneric and generic assignments of the six species treated below were made on the assumption that conchological similarity is sufficient evidence of a relationship with an odostomioid

TABLE II

Differences between two genera of eastern North American odostomioids.

Boonea

1. Adult shell >4 mm long.
2. Protoconch with $1\frac{1}{2}$ whorls, clearly heterostrophic, greatly tilted (Wells and Wells, 1961, Fig. 7).
3. Front end of foot truncate.
4. Median groove on under side of foot reaches posterior end (Figs. 30, 44).
5. Female (genital?) pore on neck.
6. Spermatophore small, stalked but not tubular, stuck in mantle cavity.
7. Massed, fresh spermatozoa faintly greyish white.
8. Spermatozoan head in a lax spiral.*
9. Fully everted proboscis about 1 to $1\frac{1}{2}$ times shell length.
10. Digestive gland cream or pale brown speckled with dark grey.
11. Parasitic on mollusks, with host preferences but no specificity; populations can be dense.
12. Shell sculpture: noded (*B. seminuda*), spirally grooved or smooth (*B. bisuturalis*), spirally grooved (*B. impressa*).

Fargoa

1. Shell <4 mm long.
2. Protoconch with ca. 1 whorl, slightly heterostrophic, untilted (Wells and Wells, 1961, figs. 3, 5).
3. Front end of foot medially cleft (Figs. 74–76).
4. Median pore or groove near posterior end of under side of foot does not reach posterior end.
5. Female (genital?) pore posterolateral to right side of mentum.
6. Spermatophore large, tubular towards unattached end, attached to last whorl of shell.
7. Massed, fresh spermatozoa pale pink.
8. Spermatozoan head linear or ovately triangular.*
9. Fully everted proboscis about twice shell length (*F. bushiana*?).
10. Digestive gland brownish grey or black.
11. Host-specific to *Hydroides* (serpulid polychaetes) [host of *F. bushiana* unknown]; populations always sparse.
12. Shell sculpture: noded (*F. dianthophila*), noded and spirally grooved (*F. bushiana*), smooth (*F. bartschi*).

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* Great variation within one species (Höisaeter, 1965, p. 65, fig. 2b-f) was not observed.

generic type-species. This assumption becomes questionable in view of the conchological convergences and divergences shown below—even in sympatric species. Understanding of evolutionary relationships is not helped by assignment of an east American species to a taxon with a type-species that is widely allopatric and unknown biologically. Thus, I have excluded further consideration of such taxa.

Table II shows that there are major biological differences between two groups of east North American odostomioids. The concept of *Fargoa* Bartsch, 1955, can be extended to cover one group. For the other, a new genus has to be named. For the first time in pyramidellid systematics, non-conchological characters are used (except for shell size and protoconchs).

SYSTEMATIC TREATMENT

Boonea Robertson, new genus

Type-species. *Jaminia seminuda* C. B. Adams, 1839.
Species also included. *Turritella bisuturalis* Say, 1822; *T. impressa* Say, 1822.
Etymology. Named for Constance (Mrs. Hollis Q.) Boone of Houston, Texas,

who for many years has been sending me both living and preserved mollusks. Gender feminine.

Differentiae. Differences from *Odostomia, sensu stricto* are given in Table I and from *Fargoa* in Table II. Shell sculpture is various (Figs. 3–5).

Boonea seminuda (C. B. Adams, 1839)

Shell. Fig. 3.

Taxonomy of species. Synonyms include *"Odostomia" willisi* Bartsch, 1909 (p. 99, pl. 13, fig. 42) and *"O." toyatani* Henderson and Bartsch, 1914.

Prior generic assignments. *"Odostomia" seminuda* has usually been classified in the subgenus *Chrysallida* Carpenter, 1857 (Bartsch, 1909, p. 97; Wells and Wells, 1961, pp. 149–150; Abbott, 1974, p. 292). *Chrysallida* was ranked as a genus by Thiele (1929, p. 232), Brandt (1968, pp. 277–278), van Aartsen (1977) and LaFollette (1977), among others. The type-species of *Chrysallida, Odostomia torrita* Dall and Bartsch, 1909, of West Mexico, has stronger axial sculpture than any species treated here, and is unknown biologically. *"O." seminuda* was placed by Odé (1968) in the subgenus (or genus) *Menestho* Möller, 1842, the type-species of which, *M. albula* (Fabricius, 1780) of the northern North Atlantic, is without the columellar plica present in all six species treated here, and is unknown biologically.

Geographic range. Prince Edward Island, Canada, to Texas. Recorded from Rio Grande do Sul, Brazil by Rios (1975, p. 143), but Brazilian specimens loaned me by Rios are probably another species. West Indian records are also dubious.

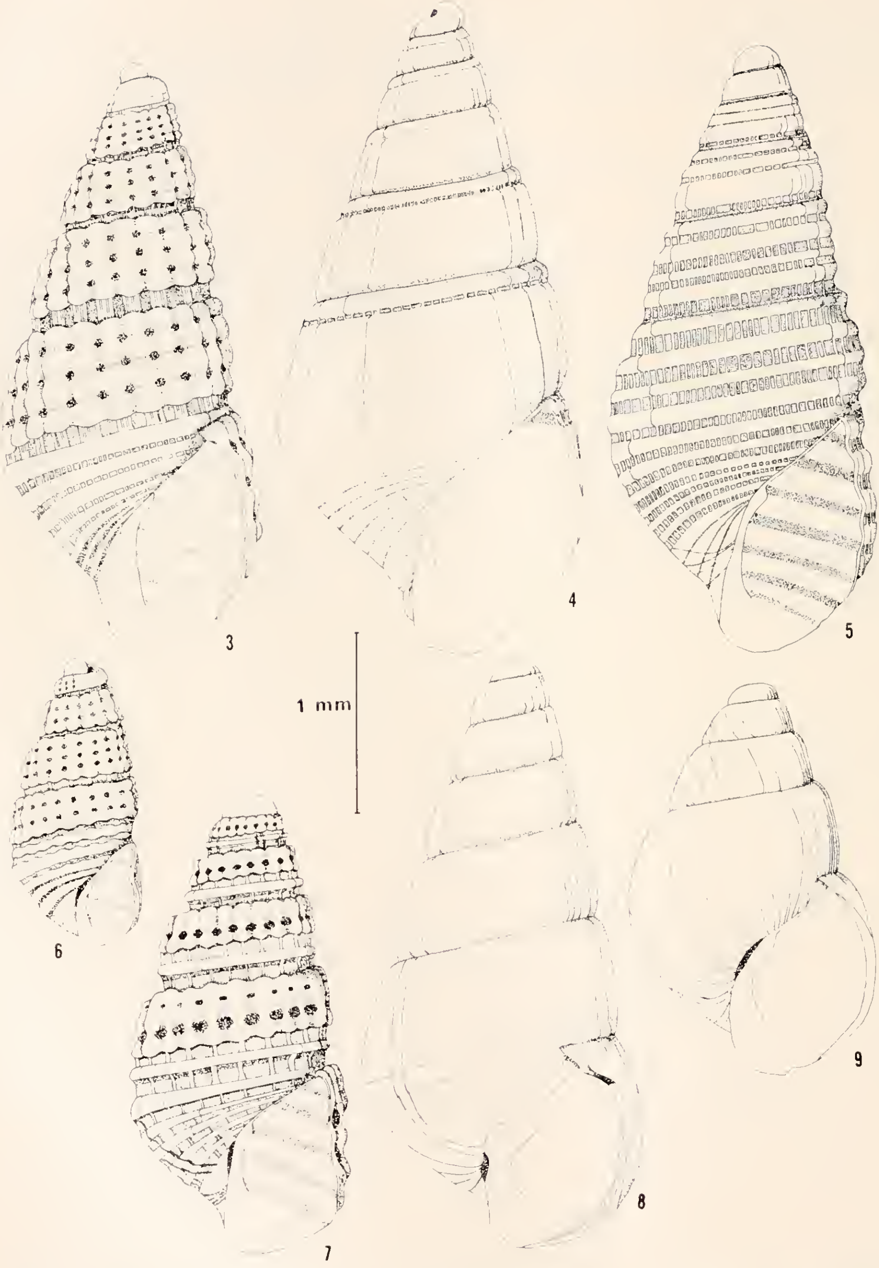
Preferred hosts. *Crepidula fornicata* (Linnaeus, 1758) [Massachusetts and North Carolina]; *Argopecten irradians* (Lamarck, 1819) [North Carolina].

Populations studied and dates of spermatophore occurrence: Massachusetts: N. end Quissett Harbor, 3 km N.E. of Woods Hole (mid-July to early August, late August to mid-September, and mid- and late October). Northwest Gutter, between Uncatena and Naushon Islands, Elizabeth Islands, 3 km S.W. of Woods Hole (early August). North Carolina: Middle Marshes, 5 km S.E. of Beaufort (on *Argopecten irradians,* early August); Wreck Point, Cape Lookout, 16 km S.E. of Beaufort (on *Crepidula fornicata,* late July, mid-August).

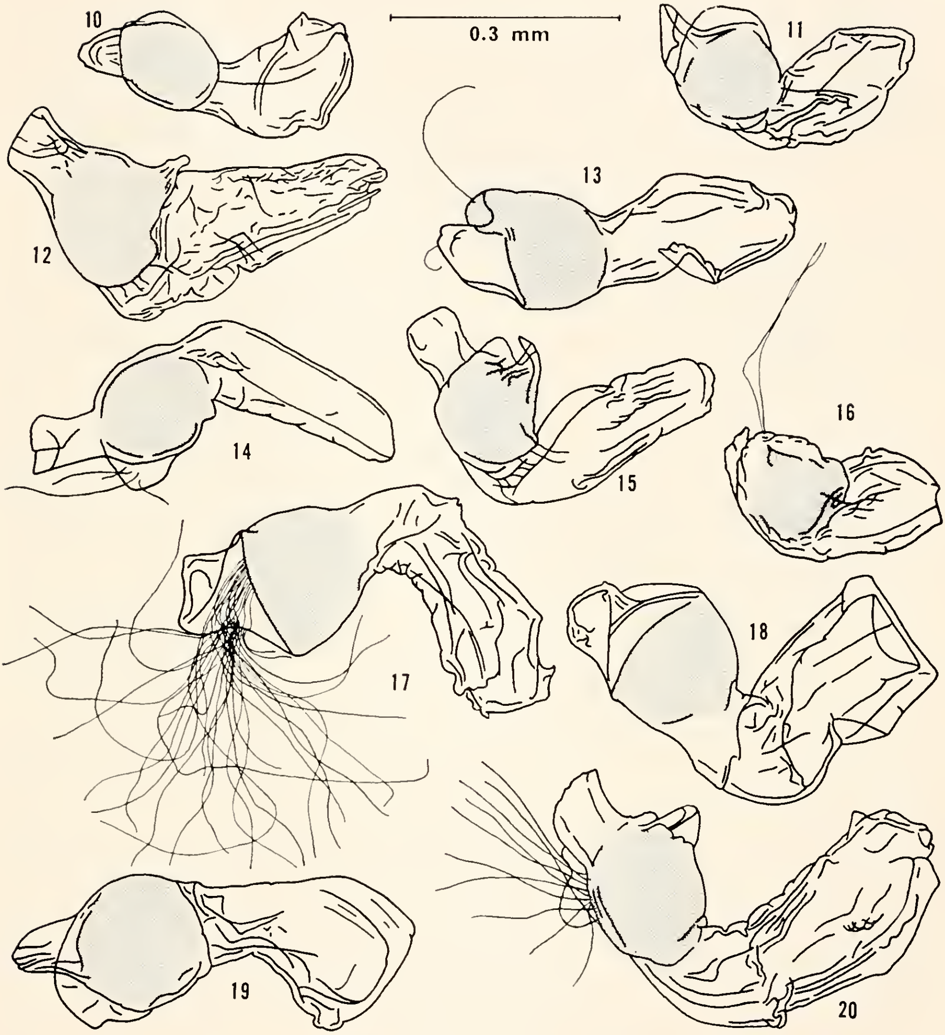
Spawning season. Massachusetts: probably mid-June to early October, but spawn was only seen in early and mid-July, early and late September, and early October. Those spawning in early October were young that had settled earlier in the same year (early summer). North Carolina: early July (Middle Marshes) and early August (Middle Marshes and Cape Lookout). Spawn was seen in January in the Beaufort area by Wells and Wells (1961, p. 156).

Spermatophore description (Figs. 10–29). Small relative to shell size (Fig. 30). No basal disc. Stalk long (rarely as short as shown on Fig. 29). Straight or bent near bulb. Bulb nearly at unattached end, with a lateral spout. Spermatozoa emerge from slit on spout. Extension beyond bulb prominent (rarely as short as shown on Fig. 16), sometimes distinguished with difficulty from spout (e.g. Fig. 17). Nowhere cuticularized. Massed, fresh spermatozoa faintly greyish white.

Transfer. This was not seen, but it probably occurs when the animals cluster and



Figures 3–9. Shells. 3. *Boonea seminuda,* North Carolina. 4. *B. bisuturalis,* Massachusetts. 5. *B. impressa,* Texas. 6. *Fargoa dianthophila,* North Carolina. 7. *F. bushiana,* North Carolina. 8. *F. bartschi,* Massachusetts (holotype). 9. *F. gibbosa,* Texas. The three species of *Boonea* (shown not full-grown) attain much larger sizes than the four species of *Fargoa.* Note that the two genera are not distinguishable by the conchological features shown here (the protoconchs differ). Drawings by Faith A. Pittman.

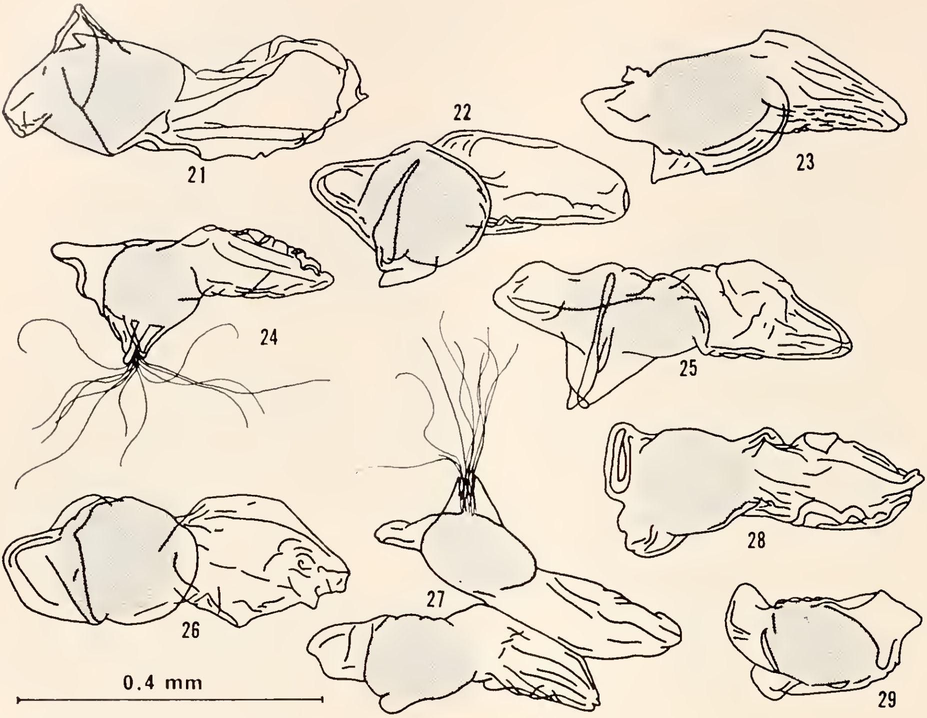


FIGURES 10–20. *Boonea seminuda* spermatophores from Massachusetts (host: *Crepidula fornicata*). Spermatozoa are seen emerging only on Figures 13, 14, 16, 17, and 20.

crawl over each other. As in *B. bisuturalis*, the spermatophores probably are positioned by the mentum and the front end of the foot.

Position. Fairly deep in the mantle cavity, consistently stuck on the right side of the rear of the neck, with the attached ends anteriad (Fig. 30). The spermatophores can occur in clusters of up to three or more (stuck together?)

Frequency. On October 27, 1977, I selected at random ten large animals from a large series collected the day before near Woods Hole. Six animals lacked spermatophores in their mantle cavities, but three had one each, and one had three clustered. The animals had been separated from their hosts, were at higher tem-



FIGURES. 21–29. *Boonea seminuda* spermatophores from North Carolina (host: *Argopecten irradians*). Figure 27 shows two stuck together by their stalks. Spermatozoa are seen emerging only on Figures 24 and 27.

peratures (> 20° C.) than in the field (15°–17° C.) and were abnormally crowded in a bowl in the laboratory. They may, therefore, have been transferring spermatophores at an unusually high rate.

Remarks. At Woods Hole, spermatozoa perhaps are stored over winter by the recipients (as in *B. bisuturalis*). Animals spawned the preceding summer and with shells less than 3.2 mm long produced spermatophores by mid-October, *i.e.*, when they were only about 3 months old. A specimen with a shell 2.4 mm long was observed to extrude a spermatophore bulb end first, indicating maturity even at this small size.

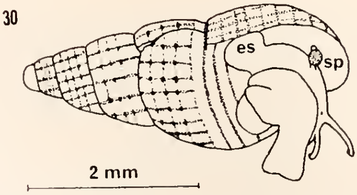


FIGURE 30. *Boonea seminuda* with a spermatophore (sp) on the neck. Note the excurrent siphon (es). From a photograph.

Massachusetts stalks commonly are bent near the bulb (Figs. 10–20), whereas those from North Carolina are nearly straight (Figs. 21–29). It is uncertain whether this difference correlates with the different locality or host.

Boonea bisuturalis (Say, 1822)

Shell. Fig. 4 (nominate form).

Taxonomy of species and shell variation. Some of the shell variation was documented by A. H. Scheltema (1965, Fig. 1A–C). Synonyms and variants include *"Odostomia" trifida* (Totten, 1834) [Bartsch, 1909, pl. 13, fig. 46], *"O." exigua* (Couthouy, 1838), *"O." trifida bedequensis* Bartsch, 1909 (pl. 13, fig. 49), and *"O." bisuturalis ovilensis* Bartsch, 1909 (pl. 14, fig. 57). Possibly hybridizes or intergrades clinally with *B. impressa,* but as shown below, their spermatophores usually differ.

Prior generic assignment. *"Odostomia" bisuturalis* has been classified in the subgenus *Menestho* (Bartsch, 1909, p. 106; Abbott, 1974, p. 295). *Menestho* was ranked as a genus by Thiele (1929, p. 234), among others.

Geographic range. New Brunswick, Canada, to vicinity of New York (erroneous or unconfirmed reports from farther south).

Preferred hosts. Various, but include *Littorina littorea* (Linnaeus, 1758) and *Crassostrea virginica* (Gmelin, 1791).

Populations studied and dates of spermatophore occurrence. Massachusetts: shore opposite Flume Pond, S. of Gunning Point, 4 km N.N.E. of Woods Hole [grooveless and multigrooved (*trifida*) forms with nominate form] (late June, late July to late October). N. end Quissett Harbor, 3 km N.E. of Woods Hole (mid-April and late October).

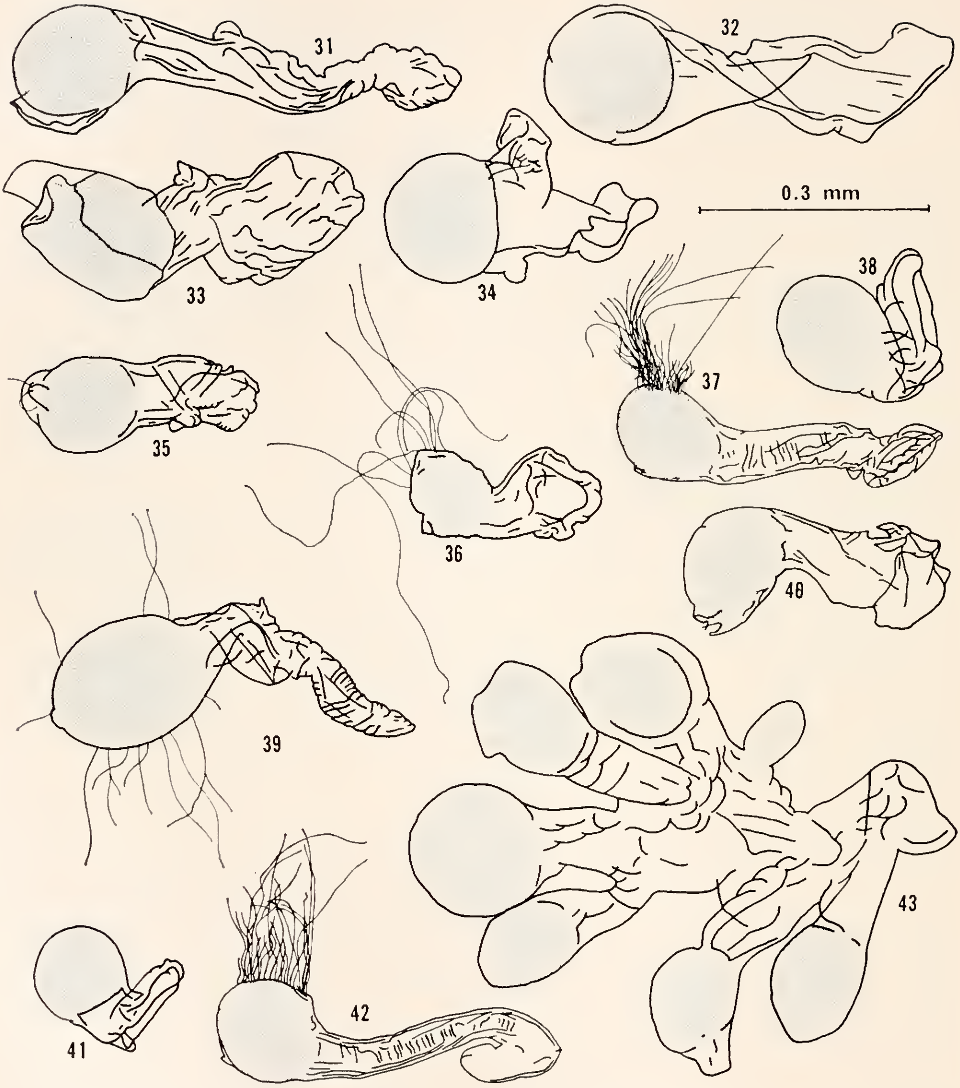
Spawning season. Early June to mid-September (numerous observations). Abnormal eggs were once seen in late September.

Spermatophore description (Figs. 31–43). Small relative to shell size (Fig. 44). No basal disc. Stalk commonly long, rarely bifurcate (Fig. 34), straight or bent near bulb. Bulb at unattached end. No spout. Spermatozoa emerge either terminally or laterally; sector of emergence usually poorly defined. Extension beyond bulb absent (the structure on Fig. 33 is anomalous; see also Fig. 43, lowest, which could be from *B. seminuda*). Nowhere cuticularized. Massed, fresh spermatozoa faintly greyish white.

Transfer. This was seen once. Two animals were adjacent, with one holding on with its foot to the basal part of the apertural side of the shell of the other. A spermatophore was first seen in transparency through the right side of the rear of the mentum. The spermatophore then was extruded bulb end first. With the aid of the anterior part of the mentum, the spermatophore was moved from beneath the mentum to the mantle cavity of the recipient, in which there already were two spermatophores.

Animals with shells 5.5 and 5.8 mm long were seen to release two fresh spermatophores from their excurrent siphons. Perhaps these spermatophores had not been properly stuck to the mantle wall.

Position. Deep in the mantle cavity, consistently stuck on the dorsal mantle wall to the left of the excurrent siphon, usually about half way between the siphon and the



Figures 31–43. *Boonea bisuturalis* spermatophores from Massachusetts. Figure 43 shows seven stuck together by their stalks. Spermatozoa are seen emerging only on Figures 35–37, 39 and 42.

anterior end of the mantle cavity; attached ends anteriad (Fig. 44). The spermatophores occur singly or in clusters of up to seven or more (Fig. 43), the stalks stuck together.

My 1967 statement about placement was erroneous, being based on spermatophores that were not fresh and had become stuck elsewhere.

Frequency. Out of ten large animals selected for study, three lacked spermatophores in their mantle cavities, two had one each, three had two each, one had four, and one had about five clustered (same date and circumstances as for *B. seminuda,* in which there were fewer spermatophores).

Remarks. At Woods Hole, spermatozoa probably are stored over winter by the recipients (compare dates of spermatophore occurrence with spawning season; I assume that the winter is too severe for spermatophores to be produced during that season). Animals spawned the preceding summer and with shells less than 3.5 mm long produced spermatophores by mid-October, *i.e.,* when they were only about three months old.

Stalk length is usually greater than in the closely related species (or geographic race?) *B. impressa.*

Boonea impressa (Say, 1822)

Shell. Fig. 5.

Taxonomy of species. Synonyms include *"Odostomia (Menestho)" beauforti* Jacot, 1921. See under *B. bisuturalis* regarding the distinctness of that species.

Prior generic assignment. "Odostomia" impressa has been classified in the subgenus *Menestho* (Bartsch, 1909, p. 103; Abbott, 1974, p. 294). *Menestho* was ranked as a genus by Thiele (1929, p. 234), among others.

Geographic range. Doubtfully Massachusetts; New Jersey to Quintana Roo, Mexico.

Preferred host: Crassostrea virginica.

Populations studied and dates of spermatophore occurrence. North Carolina: opposite Pivers Island, Beaufort (mid-August and mid-November). W. side Banks Channel, Wrightsville Beach, 14 km E.S.E. of Wilmington (mid- and late May). N.W. Florida: Dixon Bay, Wakulla County (early November). Texas: off 61st St., Offats Bayou, inlet of Galveston West Bay (mid-November, late December, early January, late February, early March and late April).

Spawning season. North Carolina: spawn seen late May and mid August. Wells (1959, p. 141) observed spawn in early May to mid-September in the Beaufort area. Texas: spawn seen late November to late December, and early March to early April.

Spermatophore description (Figs. 45–67). Very small or small relative to shell size. No basal disc. Stalk usually short and bent, uncommonly with lateral projection

44

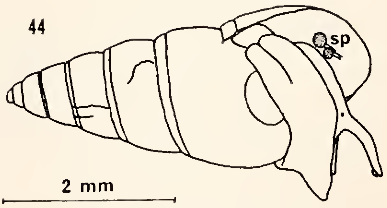
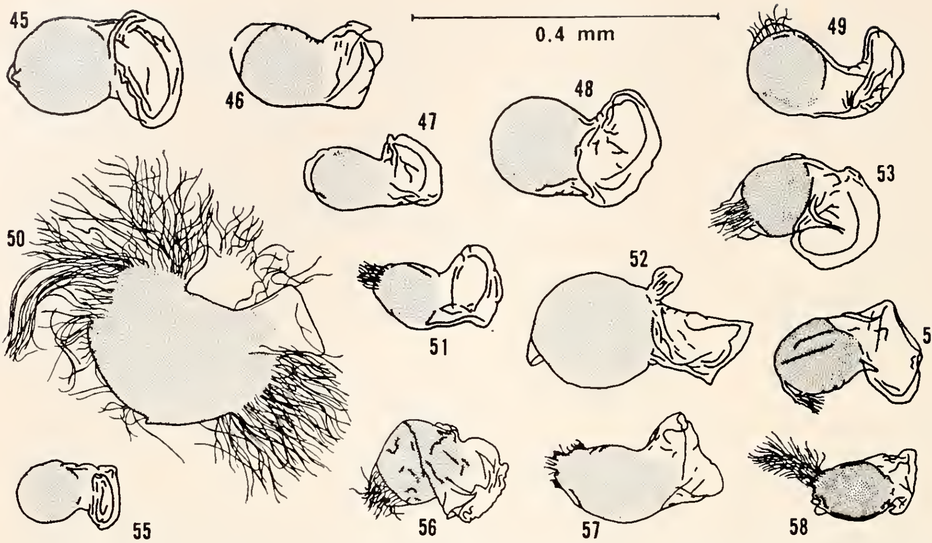
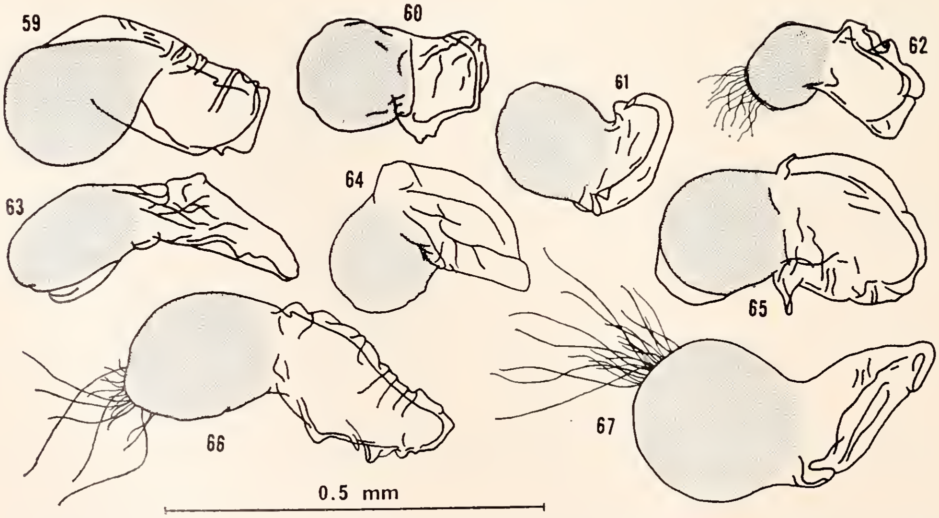


FIGURE 44. *Boonea bisuturalis* with two spermatophores (sp) on the dorsal mantle wall. From a photograph.


45
46
0.4 mm
49
47
48
53
50
52
51
54
55
56
57
58

Figures 45–58. *Boonea impressa* spermatophores from North Carolina (Figs. 45–55) and N.W. Florida (Figs. 56–58). Figure 50 was squashed more than the others. Spermatozoa are seen emerging only on Figures 49–51, 53, 54, 56–58.

(Figs. 52, 65). Bulb at unattached end. No spout. Spermatozoa emerge either terminally or laterally; sector of emergence usually poorly defined. Extension beyond bulb absent. Nowhere cuticularized. Massed, fresh spermatozoa faintly greyish white.


59
60
62
61
63
64
65
67
66
0.5 mm

Figures 59–67. *Boonea impressa* spermatophores from Texas. Spermatozoa are seen emerging only on Figures 62, 66, and 67.

Transfer. Not seen; comments under *B. seminuda* apply.
Position. Deep in the mantle cavity, consistently stuck on the dorsal mantle wall to the left of the excurrent siphon, usually about half way between the siphon and the anterior end of the mantle cavity; attached ends are anteriad. The spermatophores occur singly (four observations) or in clusters of up to four (one observation) or more; up to two were seen stuck together by their stalks.
Remarks. The North Carolina and N.W. Florida spermatophores (Figs. 45–58) are smaller than the ones from Texas (Figs. 59–67). The maximum North Carolinian and Floridian bulb diameters are 80–160 μm (average of 13 measurements [excluding Fig. 41]: 114 μm); the Texan diameters are 120–215 μm (average of nine measurements: 170 μm). Some of the Texan stalks (*e.g.,* Figs. 63 and 66) approach those of *B. bisuturalis* in length. Note that in *B. impressa* the spermatophores are placed as they are in *B. bisuturalis,* not as in *B. seminuda.*

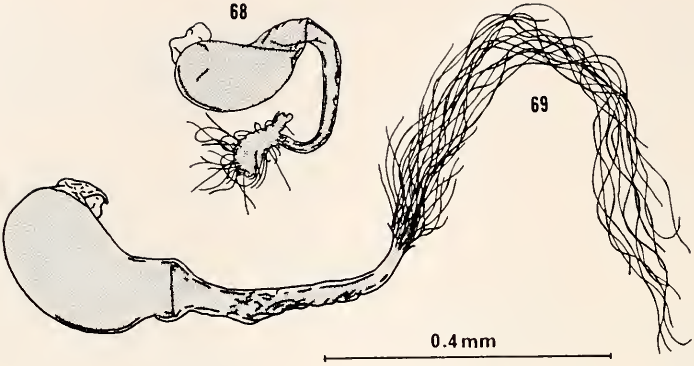
Genus *Fargoa* Bartsch (1955, p. 80)

Type-species (by original designation). *Fargoa calesi* Bartsch, 1955 ("Pliocene" or Pleistocene) = "*Odostomia*" *bushiana* Bartsch, 1909.
Species also included. "*Pyramidella* (*Sulcorinella*)" *bartschi* Winkley, 1909; "*Odostomia* (*Chrysallida*)" *dianthophila* Wells and Wells, 1961; "*Odostomia*" *gibbosa* Bush, 1909. Shell form and sculpture are various (Figs. 6–9).

Fargoa dianthophila (Wells and Wells, 1961)

Shell. Fig. 6.

Prior generic assignments. "*Odostomia*" *dianthophila* was originally classified in the subgenus *Chrysallida* (Wells and Wells, 1961, p. 152; Abbott, 1974, p. 293), but Odé (1971) placed it "in the *Menestho* group of odostomias . . . in the genus *Fargoa* of Bartsch." *Chrysallida* and *Menestho* have each been ranked as a genus (see earlier).
Geographic range. Massachusetts to Texas.
Preferred hosts. Hydroides dianthus (Verrill, 1873) [Massachusetts] and this and other *Hydroides* spp. [North Carolina to Gulf of Mexico].
Populations studied and dates of spermatophore occurrence. Massachusetts: shore opposite Flume Pond, S. of Gunning Point, 4 km N.N.E. of Woods Hole (mid-July to early August, mid-and late September, and mid-October). North Carolina: opposite Pivers Island, Beaufort (early July).
Spawning season. Massachusetts: spawn seen in mid- and late July and mid-August. North Carolina: probably spawns in late May to July in the Beaufort area (Wells and Wells, 1961, p. 156).
Spermatophore description. (Figs. 68–69). Very large relative to shell size (would not fit in mantle cavity). Basal disc present. Stalk at attached end short, with no "elbow". Bulb moderately large, swollen, without (Fig. 68) or with (Fig. 69) a secondary swelling towards the unattached end, merging with long, fairly narrow tube. Nowhere cuticularized, and unattached end not hooked or barbed. Spermatozoa emerge terminally. Massed, fresh spermatozoa pale pink.
Transfer. This was seen once. The donor crawled methodically over the shell of of the recipient, probing with its mentum. The donor then assumed the position



Figures 68–69. *Fargoa dianthophila* spermatophores from Massachusetts. Spermatozoa are seen emerging from both.

shown in Figure 70 while holding the basal disc of the spermatophore against the shell with the front end of the foot for about two minutes.

Position. Attached to the shell; basal disc on the apertural side of the last whorl, posterior and to the right of the posterior end of the aperture; unattached end anteriad, on the right side of the body, adjacent to, and then inserted deep in the female pore. One spermatophore is normally placed at a time, but one animal was seen with two spermatophores inserted in the female pore; one of them was attached also to the shell.

Remarks. These spermatophores closely resemble those of "*Chrysallida*" *interstincta* described and illustrated by Höisaeter (1965), differing only in having a

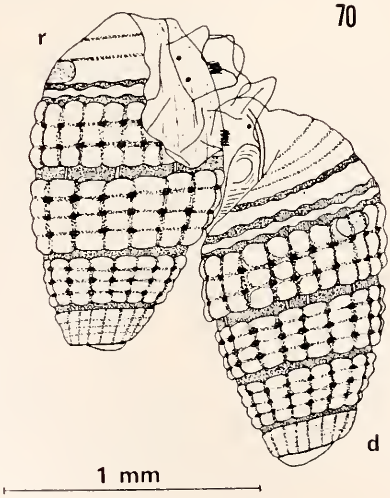


Figure 70. *Fargoa dianthophila:* positions assumed during spermatophore transfer, with the donor (d) on the shell of the recipient (r). It is difficult to see what happens. Drawing by Faith A. Pittman.

more inflated bulb and a narrower tube. Their positions on the shells are identical, but this is probably true of all the shell-attached pyramidellid spermatophores.

Fargoa bushiana Bartsch, 1909

Shell. Fig. 7.

Taxonomy of species. *F. bushiana* has commonly been known as "*Odostomia*" *dux* Dall and Bartsch (1906, p. 350, pl. 17, fig. 4; type-locality: Japan), since Wells and Wells (1961, pp. 150–151) argued that the type-locality was wrong. However, they did not see the type or other specimens identified by Dall and Bartsch, and Saurin (1962, p. 250; Saurin, 1959, pl. 5, fig. 17) recorded a range extension of "*Miralda*" *dux* to Viet Nam and the Gulf of Thailand. There could be convergence between odostomioid shells from east America and the Far East, and I prefer to use a name definitely pertaining to the American species—even though it was poorly illustrated by Bartsch (1909, pl. 13, fig. 44). Synonyms include *F. calesi* Bartsch, 1955 (type-species of *Fargoa*) and *F. archeri* Bartsch, 1955. "*Odostomia bushiana* Jeffreys, 1884" of Bush (1909, p. 482) is *Turbonilla bushiana* Verrill, 1882, synonymized by Jeffreys (1884, p. 357) with an "*Odostomia*" (actually a *Turbonilla*).
Prior generic assignments. "*Odostomia*" *bushiana* has usually been classified in the subgenus *Chrysallida* (Wells and Wells, 1961, p. 150; Abbott, 1974, p. 293), and *Chrysallida* has also been ranked as a genus (see earlier). The species has also been placed in the subgenus (or genus) *Menestho* (Odé, 1968) and in the genus *Miralda* A. Adams, 1863 (Odé and Speers, 1972, p. 9, fig. 4). *M. diadema* (A. Adams, 1860), the type-species of *Miralda*, is from Japan and is unknown biologically.
Geographic range. Massachusetts to São Paulo, Brazil (Rios, 1975, p. 143). Brazilian specimens loaned me by Rios are definitely this species.
Host(s). Unknown.
Population studied and date of spermatophore occurrence. North Carolina: Wreck Point, Cape Lookout, 16 km S.E. of Beaufort (mid-August).

Spawning season. Unknown.

Spermatophore description (Fig. 71). Very large relative to shell size (would not fit in mantle cavity). Basal disc present. Stalk fairly short, with no "elbow". Bulb fairly small, hardly swollen, confluent with wide and long tube towards unattached end. Terminal end of tube cuticularized, hooked, with a small barb. Spermatozoa emerge terminally. Massed, fresh spermatozoa pale pink.

Transfer. Not seen.

Position. Attached to the shell; the basal disc on the apertural side of the last whorl, posterior and to the right of the posterior end of the aperture; hooked end on the right side of the body, bent slightly to the right over the outer lip of the shell (the only misplaced spermatophore seen in any of the species?) The spermatophore was not seen hooked in the female pore, but this must occur.

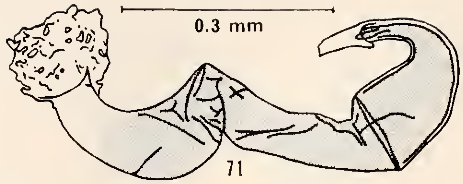


FIGURE 71. *Fargoa bushiana* spermatophore from North Carolina.

Remarks. Only one spermatophore was seen, attached to a shell only 1.4 mm long. Its unattached end is reminiscent of the cuticularized and hooked penis tips discovered by Maas (1964).

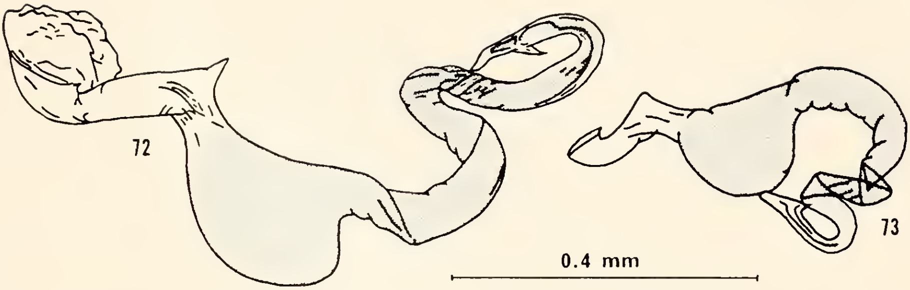
Fargoa bartschi (Winkley, 1909)

Shell. Fig. 8.

Taxonomy of species. This little-known species has been known as "*Odostomia*" *modesta* [*Chemnitzia modesta* Stimpson, 1851 not *C. modesta* Orbigny, 1842] and "*O.*" *dealbata* (Stimpson, 1851) [a *nomen dubium*]. Abbott (1974, p. 292) suggested that "*O.*" *modesta* is *O. conoidea* (Brocchi, 1814) but this European species differs. "*O.*" *modesta* of Bartsch (1909, p. 108, pl. 13, fig. 50) is "*O.*" *gibbosa* Bush, 1909 (Fig. 9). The holotype of *Pyramidella* (*Sulcorinella*) *bartschi* Winkley (1909, fig.), at the Museum of Comparative Zoölogy (no. 32810), shows that this was incorrectly described and illustrated; the outer lip is broken back and there is no deep peripheral sulcus (Fig. 8). The shell was better illustrated by Bush (1909, fig. 6). *F. bartschi* resembles the grooveless form of *B. bisuturalis* but differs in the characters shown in Table I, in having a dark, brick red, pigmented mantle organ instead of a yellow and brown one, in having a projecting excurrent siphon, and in having brown superficial spots behind the head, golden yellow spots elsewhere, and an operculum with a spiral sector that, superficially, is cream white.
Prior generic assignments. "*Odostomia*" *bartschi* has been classified in the subgenus *Odostomia* Fleming, 1813, *sensu stricto* (Abbott, 1974, p. 292, as "*O.*" *modesta*), the type-species of which, *O. plicata* (Montagu, 1803), of Europe, presumably is one of the species with penial copulation (no spermatophores) studied by Fretter and Graham (1949). The protoconch of *O. plicata* (Thorson, 1946, p. 201, fig. 115B) is unlike that of any east North American odostomioid. "*O.*" *bartschi* was also placed by Winkley (1912) in the subgenus *Evalea* A. Adams, 1860, the type-species of which, *O.* (*E.*) *elegans* A. Adams, 1860, of Japan, is unknown biologically. *Evalea* has also been classified as a "Sectio" of *Menestho* (Thiele, 1929, p. 234).

Geographic range. Massachusetts to Texas.

Preferred host. Hydroides dianthus [Massachusetts; previously unreported].
Populations studied and dates of spermatophore occurrence. Massachusetts: shore opposite Flume Pond, S. of Gunning Point, 4 km N.N.E. of Woods Hole (mid-June to early September). N. end Quissett Harbor, 3 km N.E. of Woods Hole



FIGURES 72–73. *Fargoa bartschi* spermatophores from Massachusetts (Fig. 72) and North Carolina (Fig. 73).

(mid-July to early August). North Carolina: Wreck Point, Cape Lookout, 16 km S.E. of Beaufort (mid-August). W. side Banks Channel, Wrightsville Beach, 14 km E.S.E. of Wilmington (mid- and late May).

Spawning season. Massachusetts: spawn seen mid-July to late August. North Carolina: spawn seen late May and mid-August.

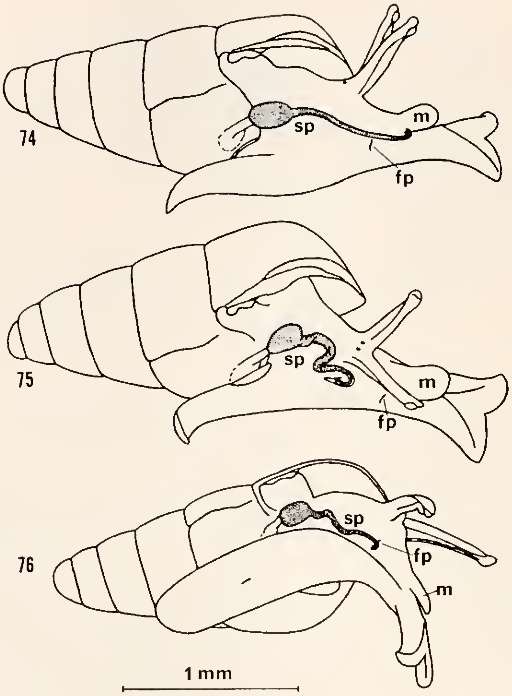
Spermatophore description. (Figs. 72–73). Very large relative to shell size (would not fit in mantle cavity [Figs. 74–76]). Basal disc present. Stalk at attached end of medium length and with a sharp lateral "elbow". Bulb on opposite side to "elbow", fairly large, distinctly swollen, fairly well demarcated from tube towards unattached end. Tube long, and unattached end cuticularized, hooked, with a fairly large barb. Spermatozoa emerge terminally. Massed, fresh spermatozoa pale pink.

Transfer. This was seen four times, once reciprocally. The donors crawled over the shells of the recipients for about five minutes or longer, with the anterior ends of the mentum and foot moving to and fro over the shell. The donors assumed the same position as seen in *F. dianthophila* (Fig. 70), with the foot wrapped around the shell of the recipient. A spermatophore was once seen emerging from beneath the eyes and under the base of the mentum, basal disc first and bulb and hooked end last. During attachment, the basal disc of the spermatophore is held against the shell with the front end of the foot for about three to ten minutes. The hooked end usually is hooked in the female pore within about one hour after transfer.

Position. Attached to the shell; basal disc on the apertural side of the last whorl, posterior and to the right of the posterior end of the aperture; hooked end anteriad, on the right side of the body, adjacent to (and then hooked in) the female pore. Usually one spermatophore is placed at a time, but twice two were seen attached near together (one was 4½ days older than the other). Sometimes the stalk breaks before the basal disc detaches, leaving one or more discs on the shell. One exceptional spermatophore remained attached to a shell for 5½ days.

Remarks. This is the most elaborate spermatophore so far known in the Pyramidellidae. Its hooked end is reminiscent of the cuticularized and hooked penis tips discovered by Maas (1964).

The spermatophores from Massachusetts (Fig. 72) were appreciably larger than any seen from North Carolina (Fig. 73). This is because the species attains much larger sizes in Massachusetts. The longest shell observed from Massa-



Figures 74–76. *Fargoa bartschi* with a spermatophore (sp) attached to the shell, first (Fig. 74) with the distal end hooked under the mentum (m), then (Fig. 75) loose, and then (Fig. 76) hooked in the female pore (fp). Figure 74 may show an abnormal occurrence.

chusetts is 4.1 mm, while the longest from North Carolina is only 1.9 mm. Spermatophores from North Carolina were observed on presumably mature shells only 1.0 and 1.3 mm long.

The statement (Robertson, 1967) about self-fertilization in *"Odostomia modesta"* is probably erroneous, having been based on a single observation of a spermatophore already attached to the shell, first with the hooked end (accidentally?) under the mentum in or near the male pore (Fig. 74), then with the unattached end loose (Fig. 75), and then later with the hooked end in the female pore (Fig. 76). It is doubtful that the animal attached a spermatophore to its own shell, and transfer was later found to occur. Thus there is no good evidence of self-fertilization in the Pyramidellidae.

Discussion

The three species of *Boonea* differ from two or more of the species of *Fargoa* in life style. The two genera have hosts in different phyla and they differ also in population density. It is the three species of *Fargoa* which are scarce and have large shell-attached spermatophores. These probably are transferred less frequently than the smaller mantle cavity spermatophores of *Boonea*. The more elaborate

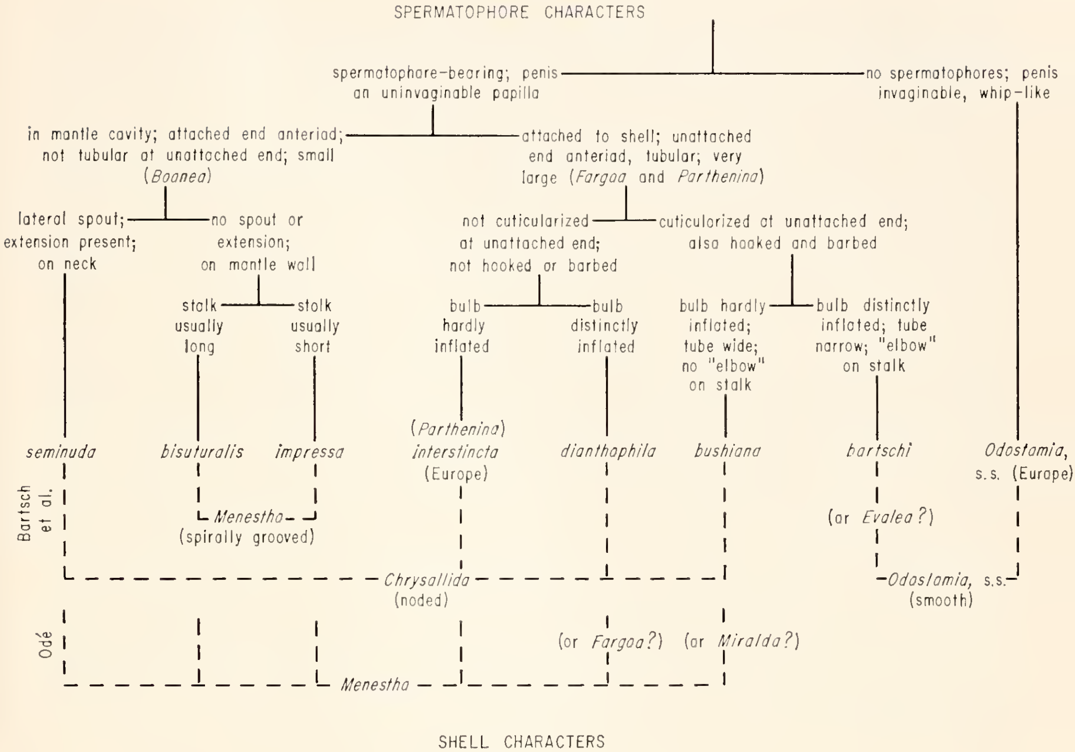

SPERMATOPHORE CHARACTERS
spermatophore-bearing; penis an uninvaginable papilla
no spermatophores; penis invaginable, whip-like
in mantle cavity; attached end anteriad; not tubular at unattached end; small (Boonea)
attached to shell; unattached end anteriad, tubular; very large (Fargoa and Parthenina)
lateral spout; extension present; on neck
no spout or extension; on mantle wall
not cuticularized at unattached end; not hooked or barbed
cuticularized at unattached end; also hooked and barbed
stalk usually long
stalk usually short
bulb hardly inflated
bulb distinctly inflated
bulb hardly inflated; tube wide; no "elbow" on stalk
bulb distinctly inflated; tube narrow; "elbow" on stalk
seminuda
bisuturalis
impressa
(Parthenina) interstincta (Europe)
dianthophila
bushiana
bartschi
Odostomia, s.s. (Europe)
Bartsch et al.
Menestha (spirally grooved)
(or Evalea?)
Chrysallida (noded)
Odostomia, s.s. (smooth)
Odé
(or Fargoa?)
(or Miralda?)
Menestha
SHELL CHARACTERS

FIGURE 77. Comparison of a classification of some odostomioids based on spermatophores with two classifications (those of Bartsch and Odé) based on shell sculpture, upon which the traditional classification into subgenera or genera is primarily based. There are also sculptural similarities between the grooveless form of *B. bisuturalis* and *F. bartschi*, and *F. bushiana* is sculpturally intermediate between *B. seminuda* and *B. impressa* (see Odé, 1968). Note that the spermatophore and two shell classifications are nonconcordant. Spermatophore characters allow a more natural classification of species than shell characters.

Fargoa spermatophores may be adapted to fertilize rarely encountered individuals, although these animals sometimes live in pairs. *Boonea* animals commonly cluster in groups.

All the east North American odostomioids may have spermatophores. The only species of these obtained alive in the present study and in which spermatophores were not found is "*Odostomia*" *gibbosa* (Fig. 9). However, only two animals of this species were under observation at different times. My prediction is that "*O.*" *gibbosa* has a shell-attached spermatophore, since the animal has the traits of *Fargoa* in Table II. The medially cleft front end of the foot (trait 3) seems to be used in positioning shell-attached spermatophores, and the female pore is in the appropriate position (trait 5) for the unattached end to hook in.

The spermatophores of the six species described above provide useful species characters. Allowing for some shell variation (such as in *B. bisuturalis*), they confirm the conchological characterizations of the species. Spermatophores support the idea that *"Odostomia" trifida* is synonymous with *B. bisuturalis,* and that this latter species is usually distinct from *B. impressa* and widely different from *Fargoa bartschi.* The data support Wells and Wells (1961) in separating three east North American species in what they called the subgenus *Chrysallida.*

The spermatophore data are significant in understanding phyletic relationships between odostomioid species—even though life style differences and correlated morphological adaptations do not necessarily parallel phyletic differences. Figure 77 shows that a classification based on spermatophores is nonconcordant with the traditional supraspecific classifications based primarily on shell sculpture (*e.g.*, see Dall and Bartsch, 1904, pp. 9–14). Which classification is closest to actual phyletic relationships? Comparison of the three species with mantle cavity spermatopheres (*Boonea*) with the three species having shell-attached spermatophores (*Fargoa*) showed that there are at least ten traits that distinguish the two genera (Table II), and difference 5 seems especially profound. No such tabulations of multiple traits can be made to support the separations and groupings based on shell sculpture. Note that conchologists disagree on how to classify some species. Since similarly-sculptured shells are separated in *Boonea* and *Fargoa,* and since each group includes dissimilarly sculptured shells, I conclude that there have been evolutionary convergences and divergences in shell sculpture. The whole supraspecific classification of pyramidellids is based on the probably fallacious premise that subjective estimates of shell similarities reflect phyletic relationships. Abbott (1974, p. 291) correctly stated that the subgenera of *"Odostomia"* are "artificial groupings." The traditional supraspecific classification of odostomioid pyramidellids is probably untenable and should henceforth be based on complexes of biological characters.

This work was supported by National Science Foundation Grant DEB 76-18835. Constance (Mrs. Hollis Q.) Boone (for whom *Boonea* is named) kindly sent live specimens from Texas, and I obtained specimens from northwest Florida collected by Jack J. Rudloe. Dr. Rudolf S. Scheltema generously provided space in his laboratory at Woods Hole, and I spent one summer at the Duke University Marine Laboratory. Dr. Catherine Thiriot-Quiévreux collected the specimens of *F. bushiana* at Cape Lookout. Dr. Eliézer de Carvalho Rios loaned specimens from Brazil. Faith A. Pittman provided patient and skillful assistance. Dr. George M. Davis, Dr. Myra Keen and Virginia Orr Maes kindly read and criticized various drafts of the manuscript.

SUMMARY

1. Six east North American odostomioid species (Gastropoda: Pyramidellidae) are shown to have spermatophores that are species-specific in structure and position. The spermatophores of *Boonea seminuda, B. bisuturalis* and *B. impressa* are stuck in the mantle cavity; those of *Fargoa dianthophila, F. bushiana* and *F. bartschi* are attached to the shell in a constant position.

2. A supraspecific classification based on spermatophores does not accord with the traditional ones based on shell sculpture. By comparing all available lines of evidence, the spermatophore classification is shown to accord more closely with phyletic relationships. Shell sculpture has converged and diverged evolutionarily and is therefore unreliable in supraspecific pyramidellid systematics. With due allowances for variation it is, however, useful at the species level.

3. The new genus *Boonea* is named, based on spermatophore and other data, with *"Odostomia" seminuda* as type-species.

4. Reasons are given for using the name *F. bartschi* for "*O. modesta*" and *F. bushiana* for "*O. dux*" of authors.

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THE LIFE-CYCLE AND TAXONOMIC RELATIONS OF *LINTONIUM VIBEX* (LINTON, 1900) STUNKARD AND NIGRELLI, 1930 (TREMATODA: FELLODISTOMIDAE)[1]

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Linton (1900) described *Distomum vibex* from the northern puffer, *Spheroides maculatus* (Bloch and Schneider), family Tetraodontidae, at Woods Hole, Massachusetts. The worms were from the pharynx, attached to the wall around the entrance to the ventral pouch. He noted that they resembled *Distomum fellis* Olsson, 1866, a species that was transferred to *Fellodistomum* Stafford, 1904 by Nicoll, (1909). Linton (1901) again reported *D. vibex* from *S. maculatus* and noted that the digestive tracts of the fishes contained small crabs, amphipods, both lamellibranch and univalve mollusks, annelids, seaweeds, and sand. This account of the food-habits was confirmed by Welsh and Breder (1922), who gave a detailed account of the life-history of *S. maculatus,* taken at Atlantic City, New Jersey. Linton (1905) reported *D. vibex* from *S. maculatus* at Beaufort, North Carolina; in this paper he described intestinal diverticula parallel to the pharynx, an error which he corrected later (1940). Linton (1910) described trematodes of fishes taken at Dry Tortugas, Florida in the summers of 1906, 1907 and 1908. It is significant that *S. maculatus* and *D. vibex* were not reported. In a review, Linton (1940) gave additional information concerning *D. vibex,* including the first and only report of the parasite in a host other than *S. maculatus*. The record of collections at Woods Hole, made primarily by Vinal N. Edwards, contains the entry (p. 42), "From oldwife, *Balistes vetula,* five, 8 September, 1903." It is the only report of infection in this host from the examinations of more than forty years. *Spheroides maculatus* is a southern species; its occurrence at Woods Hole is irregular and sporadic; it may not appear until midsummer and in 1915 Edwards recorded no *D. vibex* in the examination of 149 puffers between May and September, although in other years the incidence of infection reached 50% in July and August.

Stunkard and Nigrelli (1930) extended the description of *D. vibex*, corrected errors in Linton's accounts, and erected a new genus, *Lintonium,* for the species. The genus was assigned to the subfamily Fellodistominae Nicoll, 1909, family Fellodistomidae Nicoll, 1915; the history and naming of the family was discussed by Stunkard and Nigrelli and their decision was endorsed by Yamguti (1971, p. 69).

During June to October, 1938, 1939, and 1940, Nigrelli and Atz (1943) studied the biometry and parasites of *S. maculatus* in Sandy Hook Bay, New Jersey. They examined 294 fishes, 2 to 24 cm in length and reported on their protozoan, helminthic and crustacean parasites. Incidence of infection was correlated with date of collection, age, size, and sex of hosts. Infection with *L. vibex* increased with age of fishes and a total of 1003 specimens were taken. The authors predicated

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[1] Investigation supported by NSF DEB-74-14534-A01.

(p. 3), "*Lintonium vibex* appears to be species-specific. It has not been reported from any other fish occurring naturally in the North Atlantic." Referring to the report by Linton (1940) that *L. vibex* was found in *Balistes vetula,* they suggested that this was an accidental infection, acquired by a migrant in northern waters, since *L. vibex* had not been reported from this species in its natural habitat.

An attempt to discover the life-cycle of *L. vibex* was reported by Martin (1945). During the summer of 1938 and subsequent years he observed trichocercous, non-ocellate cercariae emerging from the bivalve mollusk, *Laevicardium mortoni.* They developed in elongate sporocysts in the haemocoelic spaces of the digestive gland. In the summer of 1940, students in the invertebrate course of the Marine Biological Laboratory found vermiform organisms in the jelly surrounding the infundibular portion of the digestive system of the ctenophore, *Mnemiopsis leidyi.* Martin recognized them as metacercariae and noted the resemblance to the cercariae from *L. mortoni.* He placed ctenophores in bowls of sea water with the cercariae. The larvae were carried into the digestive tract and moved to the infundibular region. Some shed their tails on entering the digestive tract, others on penetrating the jelly to become metacercariae, indistinguishable from those of natural infections. Martin noted that resemblance between the cercariae from *L. mortoni* and those from *Tapes decussatus,* described by Palombi (1934) as larvae of *Bacciger bacciger,* a fellodistomid species in the Mediterranean at Naples. In an attempt to infect *L. mortoni* with *L. vibex,* 21 clams which had not shed cercariae during a two-week isolation period were placed in an aquarium with eggs of *L. vibex* obtained by tearing gravid worms apart; 43 clams in another aquarium served as controls. After 23 days, a moribund experimental clam was found heavily infected with sporocysts, some containing cercariae identical with those shed by naturally infected clams. The remaining experimental clams were dissected 28 days after initial exposure to *L. vibex* eggs; one clam was infected, but with only young sporocysts containing germ balls. All the control clams were negative. Because of the inconclusive results, the experiment was repeated the next year but no infections were obtained. The larva from *L. mortoni* was described as a new species, *Cercaria laevicardium* Martin, 1945.

In the family Fellodistomidae, the first life-cycle, that of *Bacciger bacciger* (Rudolphi, 1819) Nicoll, 1914, was described by Palombi (1934). A more complete description and figure of the cercaria was given by Dolgikh (1968). The sporocysts and cercariae were found in *Chione gallica* (L. 1758) from the Black Sea. The second life-cycle in the family was reported by Chubrick (1952) for *Fellodistomum fellis.* The sporocyst and cercarial stages were found in *Nucula tenuis* in the Barents Sea. Its cercaria agrees with that of *B. bacciger* but the tail is brevifurcate and without setae, instead of non-bifid and setiferous as in *B. bacciger.* Later, Chubrick (1966) supplemented her account of the life-cycle of *F. fellis* and for *Fellodistomum furcigerum* described a cercaria so different from the larva of *F. fellis* that the identification probably is not correct.

In a consideration of cercarial structures, especially the correlation of trichocercous and furcocercous condition of tails with taxonomic relations, Cable (1954) gave a detailed description, with excellent figures, of the cercaria from *L. mortoni* which had been studied earlier by Martin (1945). The description was made from larvae obtained by dissection of clams and included the development of the excretory system. The vesicle is V-shaped, in older cercariae the arms may extend to the level of the pharynx and contain concretions. The flame-cell formula is 2 [(3 + 3) + (3 + 3)] ; a common stem extends through the length of the tail and there are two separate pores, interpreted as the relict remains of reduced furcae. Cable (1954) described the species as *Cercaria laevicardii.* The descriptions of Martin (1945) and Cable (1954) provide cardinal information on the asexual and developmental stages of the parasite in *L. mortoni.* There are successive generations of sporocysts and the cercariae emerge from the clams during both day and night. The cercaria is a tricocercous larva in which the caudal setae are arranged in fused bundles, aligned dorso-ventrally, and disposed on opposite sides of the tail, like banks of oars. These structures were described and figured by Cable (1954) as "lateral finlets." The larvae swim for about 12 hours, exhibit no photactic response, and when older may attach to the bottom of a container by the tips of their tails.

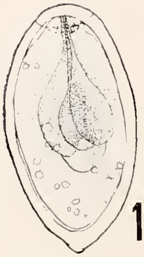
Materials and Methods

While investigating the life-cycle of *Neopechona pyriforme* (*q.v.*, Stunkard, 1969), specimens of *Mnemiopsis leidyi* were found to harbor natural infections with two different types of metacercariae: those of *N. opechona* located at the bases of the combs, and those described by Martin (1945), scattered about in the dense jelly around the infundibular portion of the digestive tract. To pursue the suggestion of Martin that *C. laevicardium* may be the larval stage of *L. vibex,* life-history experiments have been conducted as material became available.

During the summer of 1968, 246 specimens of *L. mortoni* were examined; they were collected at Waquoit, Quissett, and North Falmouth. Infections were common in clams from all three areas, and in certain collections about one-half of the specimens were infected, although after three or four weeks many of them had not liberated cercariae and infection was discovered on dissection of the clams. It is clear that under laboratory conditions, infections may be retarded or remain latent, perhaps because of inanition of the host. Specimens of *M. leidyi* were taken from the same areas as the infected clams and some were heavily infected with metacercariae of both *C. laevicardium* and *N. pyriforme.* Martin (1945, p. 206) observed, "Naturally infected *M. leidyi* had varying degrees of infection. Those collected from the Eel Pond had either very few, generally not over four, or were uninfected, while those collected from Menaunt exhibited almost 97 per cent infection, some of the infections being very heavy. The number of metacercariae per ctenophore varied in this group from 3 to well over 100."

Results

Study of the life-cycle of *L. vibex* has been continued since 1968. In former years, 1965 to 1968, puffers, *S. maculatus,* were common at Woods Hole, but in recent years the species has been rare and in certain summers none were taken until August, when a few young of the year appeared. During this period developmental stages were studied, and sketches of juvenile worms are reproduced in Figures 3 and 4. When large puffers were available as a source of worms, attempts were made to infect *L. mortoni.* Eggs were collected by placing gravid worms in small dishes of sea water for 24 hours, when those in the terminal part of the uterus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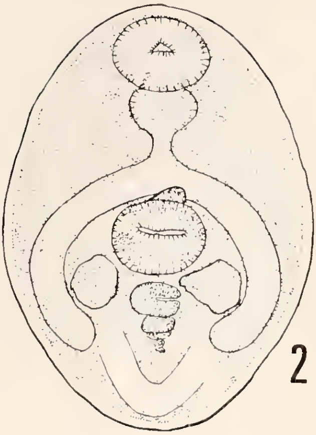
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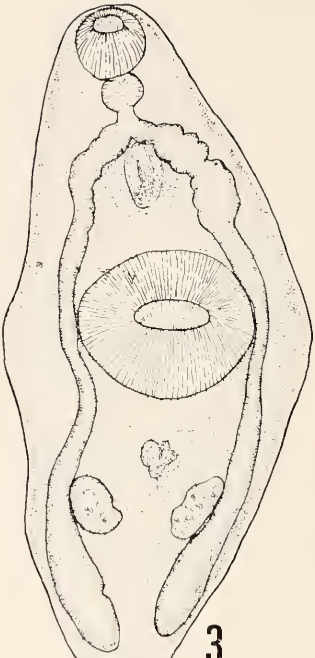
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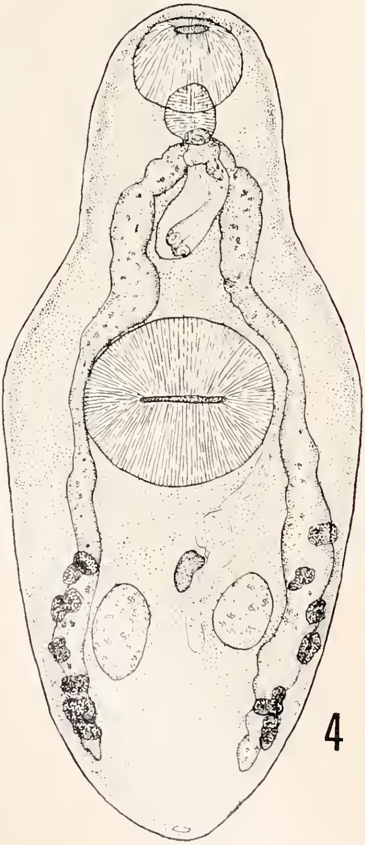
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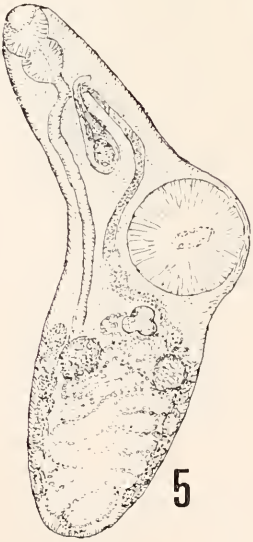
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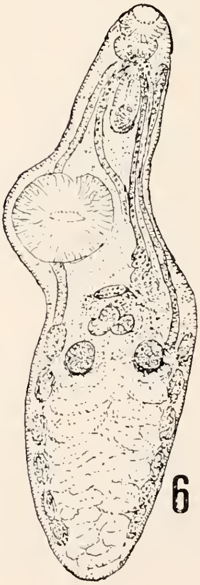
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were extruded. The eggs, (Fig. 1) measure 0.052 to 0.055 by 0.031 to 0.032 mm, have heavy yellow shells, and an operculum at the narrower end. Eggs in sea water are covered by an adhesive substance and stick to instruments, pipets, to vegetation and the bottom of a dish. Eggs from the end of the uterus appear to be embryonated, but the miracidia are motionless, except for rare and slight twitching, and hatching has never been observed. Eggs have been maintained for three weeks at summer temperature with no change in the appearance of the larvae and with no emergence. Attempts to infect *L. mortoni* by dropping eggs in the incurrent siphon were not successful and when eggs were inserted in the mantle cavity, a few shells with open opercula were found in the feces but no infections were obtained. To simulate natural conditions, clams were implanted in mud and sand in a tray, covered with a coarse wire screen and placed on the bottom of a tank that contained four large puffers. At the end of the summer, the clams were dissected with only negative results.

When attempts to infect clams proved futile, investigation turned to the infection of fishes. Maintenance of puffers in the laboratory is easy; they feed avidly and come to the surface to grab food from a pair of tweezers. To conduct infection experiments with larger fishes, it is essential to keep them for some time, preferably several weeks, to permit natural infections to mature. After feeding metacercariae, it is requisite that hosts be dissected at regular intervals to trace development of the worms and establish identity of the specimens. Scarcity of fishes prevented this type of research in some summers and recourse was taken to infection of small fishes, the young of the year. In some years they appeared early in July and in others not until the middle of August. At this time they are about 2.5 cm long and may have juvenile specimens of *Podocotyle atomon* or *Monorcheides cumingiae* in their intestines, acquired by eating *Gammarus* sp. or small bivalves that harbor the metacercariae of these species. But natural infection by *L. vibex* has never been found in these young puffers. Indeed, the ctenophores, *M. leidyi,* ordinarily do not appear in the Woods Hole area before the middle of July and sometimes not until August. When the ctenophores appear, they already harbor metacercariae of *C. laevicardium.* The young puffers appear at about the same time. They readily ingested bits of *Mytilus edulis* in which metacercariae had been implanted. In 1968 a small puffer fed metacercariae on August 2, was dissected on August 25 and a small trematode (Fig. 2) was found in the pharynx. Fixed, stained and mounted, it is 0.18 mm long, 0.11 mm wide and the oral sucker is 0.036 mm in diameter. Another fish, fed at the same time, was dissected on August 31; it contained 12

FIGURE 1. *Lintonium vibex,* egg, in sea water after three weeks at summer temperature, Woods Hole.

FIGURE 2. *L. vibex,* young worm, after three weeks in the pharynx of a young puffer of the year; taken August 25, 1968; fixed, stained and mounted, somewhat contracted, 0.18 mm long.

FIGURE 3. *L. vibex,* juvenile, natural infection, taken June 22, 1973; fixed, stained and mounted, 1.8 mm long.

FIGURE 4. *L. vibex,* juvenile, natural infection, taken June 22, 1973; fixed, stained and mounted, 2.60 mm long.

FIGURE 5. *Lintonium hetrorchis* (Bilquees, 1972) Madhavi, 1975, paratype specimen, from the U. S. National Museum, 7.2 mm long.

FIGURE 6. *Lintonium isorchis* (Bilquees, 1972) Madhavi, 1975, paratype specimen, from U. S. National Museum, 8.82 m long.

juvenile *L. vibex*. When alive, the worms were larger than when fixed, stained and mounted. In 1977, the ctenophores did not arrive until August and the feeding experiments were continued. The experiment was terminated the middle of September when the fishes were about 6 cm long. Young worms, about twice as large as the metacercariae, were recovered. The recovery of juvenile worms, three to four weeks after feeding of metacercariae, supports the postulate that *C. laevicardium* is the larva of *L. vibex*.

Discussion

The systematic relations of *L. vibex* are involved, confused, and uncertain. A review of the literature discloses the situation. Johnston (1913) described *Steringotrema pulchrum* from *Tetraodon hispidus* taken in Queensland, Australia. He noted (p. 389), "While I place this species in Odhner's genus *Steringotrema,* it appears to be more related to *Distomum vibex* Linton than to any of the three species enumerated by Odhner. *D. vibex* evidently belongs to the same genus." Odhner (1928) declared that *S. pulchrum* Johnston is identical with *Gastris consors* Lühe, 1906, a parasite of *Tetraodon stellatus* from the Pearl Banks of Ceylon, Indian Ocean. Layman (1930) reported specimens from *Cantherines modestus* taken in Peter-the-Great Bay, a portion of the Sea of Japan, and assigned them to *S. pulchrum* Johnston, 1913. Yamaguti (1934) identified specimens from *Monacantus cirrhifer* taken in Toyama Bay, Sea of Japan, and *Alutera monoceros* (= *Cantherines unicornu* Basilewsky) from the Pacific coast of Japan as *Distomum vibex* Linton, 1900. He noted the similarity with *S. pulchrum* Johnston, 1913 and suggested that the American, Australian and Japanese species of *Lintonium* may be conspecific. Although Yamaguti (1934) recognized the species, *pulchrum,* as a member of *Lintonium,* the new combination *Lintonium pulchrum* (Johnston, 1913) was made by Yamaguti (1954).

Crowcroft (1950) described specimens from the gullet of *Cantherines setosus* and *Cantherines güntheri* taken in southern Tasmania as *Lintonium vibex*. A cotype specimen of *C. pulchrum* from the Australian Museum was described as *Lintonium consors* and represented in Figure 2. It is very similar to *L. vibex;* the only difference is in the disposition of the vitellaria, and the figure might have been made from a specimen of *L. vibex* taken at Woods Hole. Crowcroft did not examine specimens of *Gastris consors* but the figure by Lühe (1906) was reproduced as Figure 1. Despite the enormous differences between the figures of *S. pulchrum* and *G. consors,* Crowcroft accepted the claim of Odhner on the identity of the two species. The name *Gastris* Lühe, 1906 was preoccupied and Crowcroft suggested the new combination, *Lintonium consors* (Lühe, 1906). He observed that *G. consors* and *S. pulchrum,* regarded as identical, are from warm waters of Ceylon and Australia and like *L. vibex* occur in fishes of the family Tetraodontidae, whereas the parasites from hosts in Japan, Peter-the-Great Bay and Tasmania occur in fishes of the family Balistidae that live in cool water. Crowcroft stated (p. 317), "it might be argued, however, that the forms most closely related to *L. vibex* from the Woods Hole region are those reported by Yamaguti (1934), Layman (1930), and in the present paper. These forms, which all occur in cool waters, are identified with *L. vibex* simply because the recorded differences, using our present criteria, are not sufficient to justify specific differentiation." Yamaguti (1954) gave a generic diagnosis of *Lintonium* Stunkard and Nigrelli, 1930 (syn. *Gastris* Lühe, 1906, *nec Gastris* Billberg, 1820), and listed three valid species: *L. vibex* (Linton, 1900) Stunkard and Nigrelli, 1930; *L. consors* (Lühe, 1906) Crowcroft, 1950; and *L. pulchrum* (Johnston, 1913) new combination.

Skrjabin and Koval (1957) recognized four valid species in the genus *Lintonium,* the three listed by Yamaguti and *Lintonium laymani* n. sp., a new designation for *Steringotrema pulchrum* of Layman (1930).

Winter (1958) redescribed and figured *L. vibex* from *Spheroides lobatus,* taken in the Bahia de los Muertos, Golfo de California, Baja California Sur, México. This report gave records of host and locality and the description extended the limits of variation in the species. Winter stated (p. 179), "Debe tenerse en cuenta que Skrjabin y Koval (1957) registraron cuatro especies válidas para el género *Lintonium: L. consors, L. vibex. L. pulchrum* y *L. laymani,* la última de las cuales ha sido llamada por el "*Steringotrema pulchrum*" de Layman (1930). Sólo las dos primeras de estas son consideradas válidas por el autor; la tercera y cuarta especies se consideran como sinónimos, de acuerdo con Crowcroft (1950). La distribución de las dos especies del género *Lintonium,* exclusivamente de peces plectognatos en las familias estrechamente relacionadas Balistidae y Tetraodontidae, es algo interesante. *Lintonium vibex* has sido reportado de ambas familias en el Atlántico Norte templado de América, de un huésped tetraódontido en el Pacífico oriental tropical de México (Golfo de California), y de la familia Balistidae en aguas templadas y de transición alrededor de Japon y en al mar de Tasmania. *Lintonium consors* es conocida solemente de la familia Tetraodontidae en las aguas cálidas de Ceilán y Queensland, Australia."

Manter and Pritchard (1962) redescribed *L. consors* on specimens from *Arothron hispidus* (syn. *Tetraodon hispidus*) in Hawaii. The worms were large 15 to 16.6 mm long and 4.3 to 5.7 mm wide. They declared (p. 115), *L. pulchrum* "is distinguished from *L. consors* by its smaller size, a hindbody about half the total length, a more posterior acetabulum, the ovary almost immediately posterior to the acetabulum, and eggs 43–48 by 32–35 μ." Further, they predicated that the specimens from *Cantherines setosus* and *C. güntheri* taken in Tasmania by Crowcroft are not *L. vibex.* The worms were regarded as specifically identical with those from *Cantherines modestus* taken in Peter-the-Great Bay and described by Layman (1930) as *Steringotrema pulchrum.* These specimens, together with those from *Cantherines unicornu* and *Monacanthus cirrhifer* assigned to *L. vibex* by Yamaguti (1934) were recognized as *Lintonium laymani* Skrjabin and Koval, 1957.

Oshmarin (1965) described and illustrated (Fig. 16), nine specimens taken October 10, 1960 from *Alutera monoceros* on the coast of North Vietnam. The worms were assigned to *Lintonium vibex.* I am indebted to Dr. B. Lebedev of the Far-Eastern Center, USSR Academy of Sciences, Vladivostok, for a reprint of the paper by Oshmarin and to the Misses Nina Root and Mildred Bobrovich of the library staff of the American Museum for an English translation of the report. The measurements of the worms agree substantially with those given by Layman (1930) and Yamaguti (1934) for worms from the same host species in the Sea of Japan, and specific identity is apparent.

Machida (1971) reported on fellodistomid trematodes from *Navodon modestus* (= *Cantherines modestus*) and *Stephanolepis cirrhifer* (= *Monacanthus cirrhifer*) from the Toushima Islands in the Sea of Japan, regarded as identical with worms from the same hosts by Layman (1930) and Lamaguti (1934). He observed (p. 190), "As Yamaguti (1934) pointed out, *vibex* and *pulchrum* resemble each other and it is very probable that they belong to one species. This applies to *laymani;* the dimensions of *laymani* with comparisons to those of the present specimens of *vibex* are shown in Table 3. There seems to be little difference between the two, and the slight differences fall within the limits of variation of *vibex*. According to the key given by Skrjabin and Koval (1957), the acetabulum is situated equatorial in *laymani* whereas it is in the anterior half of the body in *vibex*. However, the examination of the present specimens demonstrated that the situation of the acetabulum is variable so that it makes no distinction between the two. The arrangement and shape of the other organs resemble each other. Of *Lintonium* species, at least *laymani* is considered to be placed in synonymy with vibex." The report by Machida confirms the idea that the specimens described by Yamaguti (1934) are specifically identical with those of Layman, but does not establish their identity with *L. vibex*.

Bilquees (1972) erected a new genus, *Paradiplobulbus,* to contain two new species from *Tetraodon lunaris,* family Tetraodontidae, taken on the Karachi coast of West Pakistan and described as *Paradiplobulbus hetrorchis* and *Paradiplobulbus isorchis.* Madhavi (1975) reviewed the report by Bilquees and predicated (p. 270), "The genus *Paradiplobulbus Bilquees,* 1972 very closely resembles *Lintonium* from which it was not differentiated." . . . "Here it is regarded as a synonym of *Lintonium* and the two species *P. isorchis* Bilquees, 1972 and *P. heterorchis* Bilquees, 1972 from *Tetraodon lunaris* from the coast of Karachi hence become *L. isorchis* (Bilquees, 1972) n. comb. and *L. heterorchis* (Bilquees, 1972) n. comb. respectively. Another genus that seems to be identical to *Lintonium* is *Paradiplangus* Gupta, 1968. It agrees in all details with *Lintonium* except for the I-shaped excretory bladder. The two species, *P. tetradontis* Gupta, 1968 and *P. indicus* Gupta, 1968 from *Tetraodon viridipunctatus* in Madras (Bay of Bengal) agree in many respects with *L. pulchrum* collected by me from *Gastrophysus lunaris.* Additional specimens of *P. tetradontis* and *P. indicus* may well show that they are synonymous with *L. pulchrum.*"

Madhavi (1975) described ten species of fellodistomid trematodes, four of them new, from marine fishes of the Waltair coast, Bay of Bengal. He identified as *Lintonium pulchrum* (Johnston, 1913) 12 specimens found in one of 21 green, rough-backed globe fish, *Gastrophysus lunaris* (Bleeker). According to Dr. James Atz, of the American Museum, the host is *Lagocephalus lunaris* (Bloch and Schneider), family Tetraodontidae. The description was brief, cursory, and added nothing to knowledge of the species. Madhavi also described *Lintonium pseudovibex* n. sp. from three specimens found in the pig-faced leather-jacket, *Monacanthus choirocephalus,* family Balistidae. Madhavi accepted the criteria proposed by Manter and Pritchard (1962) to distinguish between *L. vibex* and *L. laymani* and observed (p. 269), "*L. pseudovibex* shows characteristics that are intermediate between *L. vibex* and *L. laymani*. The sucker-ratio approaches that of *L. laymani* almost as closely as that of *L. vibex* and the egg-size falls within the limits for

L. laymani." There appear good reasons for regarding *L. pseudovibex* as a synonym of *L. laymani*.

A further complication was introduced by the statement of Madhavi (p. 270), "A combination of sucker-ratio and egg-size similar to that in *L. pseudovibex* also occurs in specimens of *L. vibex* reported by Parukhin and Chikunova (1964) from *Abalistes stellaris* from South China Sea. Hence they are regarded as *L. pseudovibex* n. sp." I have not been able to obtain a copy of the paper by Parukhin and Chikunova, but if the specimens they identified as *L. vibex* are identical with *L. pseudovibex,* they may be *L. laymani*.

The genus *Paradiplobulbus* Bilquees, 1972 was suppressed as a synonym of *Lintonium* by Madhavi (1975). Originally, the genus was included in the family Allocreadiidae and compared only with *Diplobulbus* Yamaguti, 1934 (Fellodistomidae); *Labrifer* Yamaguti, 1936 (Lepocreadiidae); and *Brevicreadium* Manter, 1954 (Zoogonidae). The type and paratype specimens were deposited in the Helminthological Collection of the U. S. National Museum, and through the kindness of Dr. J. Ralph Lichtenfels, I have studied paratypes of both species. The paratype of *P. hetrorchis* is shown in Figure 5. The specimen is 7.2 mm long with the following measurements: acetabulum, 1.25 mm; oral sucker, 0.45 mm; pharynx, 0.40 mm; ovary, 0.33 mm; testis 0.40 by 0.47 mm; eggs, average 45 by 32 μ. Figure 6 is of the paratype specimen of *P. isorchis.* It is the one mentioned by Bilquees (p. 252) and shown in his Figures 13 and 14, as atypical in the shape and location of the testes and with an extracecal uterine coil in the preacetalular region, which partially overlies the pharynx. In this specimen, eggs have been extruded from the uterus into the tissues and the preacetabular uterine coil suggests that the worm had been subjected to temporary pressure before fixation. In Bilquees' Figure 13, the structure labelled as RS is not a seminal receptacle; it is an early uterine coil which contains spherical, thin-shelled eggs and in which the embryos are heavily stained. The specimen is 8.2 mm long, with the following measurements: acetabulum, 1.35 mm; oral sucker, 0.52 mm; pharynx, 0.45 mm; ovary, 0.38 mm; testis, 0.60 mm; eggs, average, 48 by 32 μ. The opinion of Madhavi is accepted; the worms belong to the genus *Lintonium,* and although very similar, may be distinct. Whether or not they are identical with *L. pulchrum* is uncertain.

As noted earlier, Madhavi (1975) also suppressed *Paradiplangus* Gupta, 1968 as a synonym of *Lintonium* and predicated that the two species, *Paradiplangus tetradontis* and *Paradiplangus indicus* may be identical with *L. pulchrum.* It is significant that Madhavi did not cite a reference to the publication by Gupta (1968) on the new genus *Paradiplangus* with the new species, *P. tetradontis* and *P. indicus.* The description of *Paradiplangus indicus* sp. nov. was published in the *Riv. Parassitol.,* 29: 17–20, (1968), and the references in that paper include the entry, "Gupta, A. N. (1968): Studies on a new genus *Paradiplangus* and a new species *P. tetradontis* (Trematoda-Digenea) from *Tetradon viridipunctatus* (Günther) from Puri, India. *Rev. Biol. Trop. Costa Rica* (in press)." An examination of the *Rev. Biol. Trop. Costa Rica* for the period 1967–1974 did not disclose the reported publication and it appears certain that the genus was not described there. No other information is available here. *Paradiplangus indicus* was based on two specimens, recovered from the dissection of 20 *T. viridipunctatus.* The description is brief and

Figure 1 is of a distorted, contracted, flattened worm, that provides little specific information. The excretory vesicle was described as tubular, which, if correct, would exclude the species from the family Fellodistomidae. Indeed, Gupta included the genus *Paradiplangus* in the family Callodistomidae.

Yamaguti (1971) accepted *L. vibex* (Linton, 1900); *L. consors* (Lühe, 1906); *L. pulchrum* (Johnston, 1913); and *L. laymani* as valid species. He stated that *L. novikovi* Baeva, 1965 does not belong in *Lintonium,* but made no allocation of the species. The distinctive features of the named species are uncertain and the extent to which morphology is influenced by host and environmental conditions can not be determined with assurance. Knowlege of life-cycles, intermediate hosts, and developmental stages will be of value in specific diagnosis. Meanwhile, the criteria that have been employed in specific determination are variable, flexible, and must be applied with discretion. The worms are blood-suckers and occur in small, never large numbers. They may live for many months, possibly a year or more, and grow as long as they live. They become gravid at one-third of the maximum size and growth of regions and organs is not constant, but varies with age and size; possibly also with the host. Sucker-ratios, the relative size of oral and acetabular suckers, are significant, but the size of the sucker is dependent on the amount of pressure exerted on the structure during fixation. Egg-size is also significant but young worms, especially, produce eggs of differing sizes. In the initial portion of the uterus the eggs are broadly oval, almost spherical, and the shells are thin, flexible, almost transparent. As they traverse the uterine coils to the posterior end of the body and forward to the metraterm, they become smaller and narrower, the shells contract, thicken and become yellow or brown.

Lintonium pulchrum is very similar to *L. vibex* and the two may be identical, as suspected by Johnston. The studies of Manter and Pritchard established the validity of *L. consors.* The specimens described by Crowcroft (1950), Parukhin and Chicunova (1964), Oshmarin (1965) and Machida (1971) as *L. vibex* and those described by Madhavi (1975) a *L. pseudovibex,* may belong to *L. laymani.* The specimens described by Bilquees (1972) as members of a new genus and transferred to *Lintonium* by Madhavi (1975) probably belong to distinct species and study of additional material should clarify the situation.

SUMMARY

Distomum vibex was described by Linton (1900) from *Spheroides maculatus* at Woods Hole, Massachusetts; the species was named type of a new genus, *Lintonium,* by Stunkard and Nigrelli (1930). Sporocysts and cercariae were described and the life-cycle was postulated by Martin (1945); further detailed descriptions of the sporocysts and cercariae were provided by Cable (1954), and a report of the life-cycle is given in the present paper. Other species, assigned to the genus *Lintonium,* have been described from the Sea of Japan, the South China Sea, Tasmania, Australia, Ceylon, the Bay of Bengal, West Pakistan, Hawaii, and the Gulf of California on the west Coast of Mexico. Published accounts are reviewed, but possible influence of development in different hosts can not be assessed with confidence and criteria for specific determination are uncertain. When information on life-cycles and intermediate hosts becomes available, taxonomic decisions can be made with more assurance.

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MICROSPECTROFLUORIMETRIC MEASUREMENTS ON CELLS CONTAINING BIOGENIC AMINES IN THE CEREBRAL GANGLION OF THE POLYCHAETE *NEREIS VIRENS* (SARS)

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The cerebral ganglion of nereid polychaetes performs both neural and endocrine functions. The latter role is poorly documented but behavioral studies (Evans, 1969; 1973) indicate the involvement of the cerebral ganglion in modulation of behavior patterns as well as the importance in behavior of the integration of sensory input from varied prostomial sensory structures (Evans, Cram and Rogers, 1974). The morphology of this ganglion (Holmgren, 1916) indicates massive sensory connections with the anterior and middle levels of the brain, and although neurosecretory elements have not been precisely localized (Marsden, 1978), there is both experimental and histochemical evidence in support of the hindbrain as a primary site of endocrine activity (Clark and Olive, 1973). There is, therefore, a relative wealth (among polychaetes) of information on the nereid cerebral ganglion in terms of microstructure and the kinds of function performed, but very little indeed is known about the biochemical basis of neural activity in this or any other part of the polychaete nervous system.

The nereid cerebral ganglion should, therefore, be a good place to begin studies on the biochemical parameters of the polychaete brain. In this study, on the cerebral ganglion of *Nereis virens,* we have looked for biogenic amines, known to serve as neurotransmitters in many animals, using the techniques of both fluorescence microscopy and microspectrofluorimetric analysis. Previous reports of catecholamines and/or indolylethylamines in the polychaete central nervous system are based on biochemical and fluorescence studies only. They present somewhat varied evidence and, since the optical systems used have not always been clearly defined, the results, in some cases, are hard to evaluate. Microspectrofluorimetric measurements, the surest way of defining the nature of a biogenic amine, have not previously been published for polychaetes.

MATERIALS AND METHODS

Animals

Nereis virens was obtained from Marine Research Associates, St. Andrews, New Brunswick and was acclimatized to sea water at 11° C. Animals of medium size (3–5 g) were used.

Sections and staining

Paraffin sections, for comparison with the frozen sections used in fluorescence studies, were prepared from prostomia fixed in Bouin's fluid and stained with cresyl violet, paraldehyde fuchsin or Bodian's protargol silver (Pearse, 1968). As a preliminary step in the localization of the amine-containing cells, 8 living ganglia were stained for 15 minutes at 23° C in a 0.01 mg/ml solution of neutral red in sea water, a vital dye accumulated selectively by aminergic neurons (Stuart, Hudspeth and Hall, 1974).

For fluorescence studies, the prostomium was removed from each of 25 animals anaesthetized in MS222, immediately mounted on a cryostat objective, with O.C.T. compound, placed in commercial propane immersed in liquid nitrogen for 15 to 20 seconds, and then placed in an American Optical Model 840C cryostat set at −35° C. Freezing in liquid nitrogen alone was also tried; no differences in the quantity, discreteness or nature of the fluorescence were seen. Trays of $CaCl_2$ were stored in the cryostat, maintaining a relative humidity between 0 and 30%. Alternate sections (10–12 μ) were used for fluorescence study and as controls. Sections for fluorescence study were kept over P_2O_5 in a closed container in the cryostat for 3 to 48 hours and were then immediately placed on a warming plate to avoid water uptake. They were then kept at 80° C for an hour in a closed jar containing 0.5 g of paraformaldehyde that had been equilibrated in a desiccator over H_2SO_4 at a relative humidity of 50 to 60% (Hamberger, Malmfors and Sachs, 1965). Control sections were treated in the same way except that they were heated in an environment free of formaldehyde vapor. After examination for autofluorescence, control sections were dehydrated and stained with cresyl violet. Freeze-drying of entire prostomia or of supraoesophageal ganglia attached to connective tissue, followed by vacuum embedding in paraffin, was attempted, but abandoned after repeated failure to obtain intact sections. Difficulties with freeze-drying of marine tissues are well known (Welsh, 1972; Stuart *et al.*, 1974).

Microscopy and microspectrofluorimetry

All slides were examined and photographed for specific fluorescence within 24 hours of formaldehyde treatment. Analysis of color and emission spectra of specific fluorescence was carried out on a Leitz Orthoplan microscope equipped with a vertical illuminator consisting of a Ploempak 2 turret system with three combinations of built-in excitation filters, a dichroic-beam splitting mirror and suppression (barrier) filters matched to them (Ploem, 1971). A combination of excitation filter BG3 and S405, dichroic mirror TK 455 and suppression filter K460 gave the best separation of colors, resulting in a cyan (blue-green) for catecholamines and a yellow for indolylethylamines. The filters selected provided excitation energy, with a maximum around 410 nm, reaching the specimen as well as transmission of wave lengths emitted by the specimen above 460 nm. Sometimes a combination of excitation filters BG 12 and KP 5000, dichroic mirror TK 510 and suppression filter K 515 was used for comparison with fluorescence characteristics reported by other authors (Rude, 1966; Myhrberg, 1967). The light source was a high pressure mercury lamp (Phillips CS 100-W2) with a voltage stabilizer.

Emission spectra were measured with an improved Leitz MPV1 unit consisting of the orthoplan microscope as described above as well as an EMI 9558-B photo multiplier tube (S20 cathode) connected to an ultrastabilized high voltage supply, type NSHM BN (Knot Elektronik, München). A Leitz 100× objective and 10×

eyepiece together with a 0.25× projective were used and measurements were made with an aperture of 15 μ^2. A Veril B60 interference graduated filter (Jenaer Glaswerk, Schott and Gen., Mainz, Germany) installed in the measuring beam was used for spectral analysis and a galvanometer recorded intensity. Measurements of photodecomposition (decay of specific fluorescence with exposure to UV light) were taken with the interference filter set at the emission maximum of the fluorescence concerned. The spectral sensitivity of the system was calibrated against a stabilized halogen lamp of known spectral characteristics. Within the spectral range used in this study there was no need to correct emission measurements. As a further check on the reliability of the system, the emission spectrum of a narrow band Hg line 546-10 Schott Model filter was measured once a week. Model droplets of standards were prepared by dissolving 5-HT creatine sulphate and noradrenalin bitartrate in a 2% solution of bovine serum albumin 0.15 M potassium phosphate buffer to a concentration of 0.002 M (Björklund, Nobin and Stenevi, 1971). The solutions were sprayed as droplets on glass slides, dried at room temperature, exposed to formaldehyde and examined by microspectrofluorimetry. Measurements obtained were compared with published spectra for these compounds.

When a reading was taken, the field diaphragm of the illuminating beam was closed until it just surrounded the area of the specimen so that fluorescence emitted by adjacent structures was eliminated. The interference filter was passed across

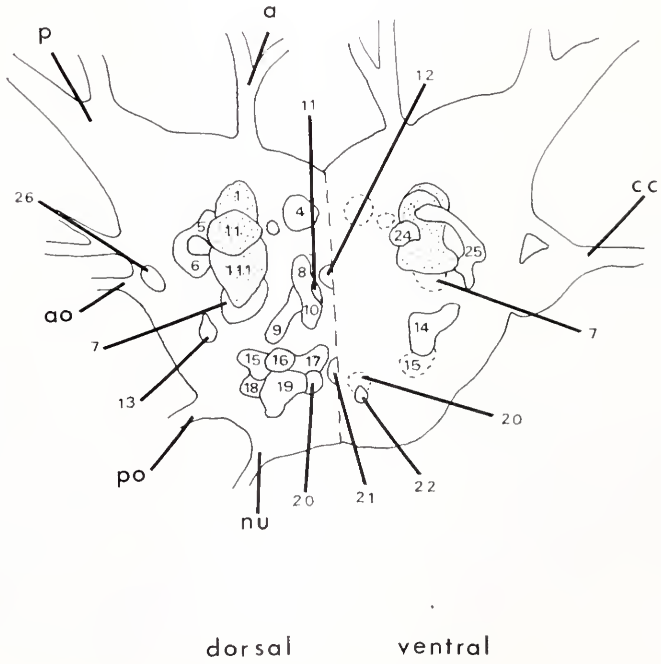
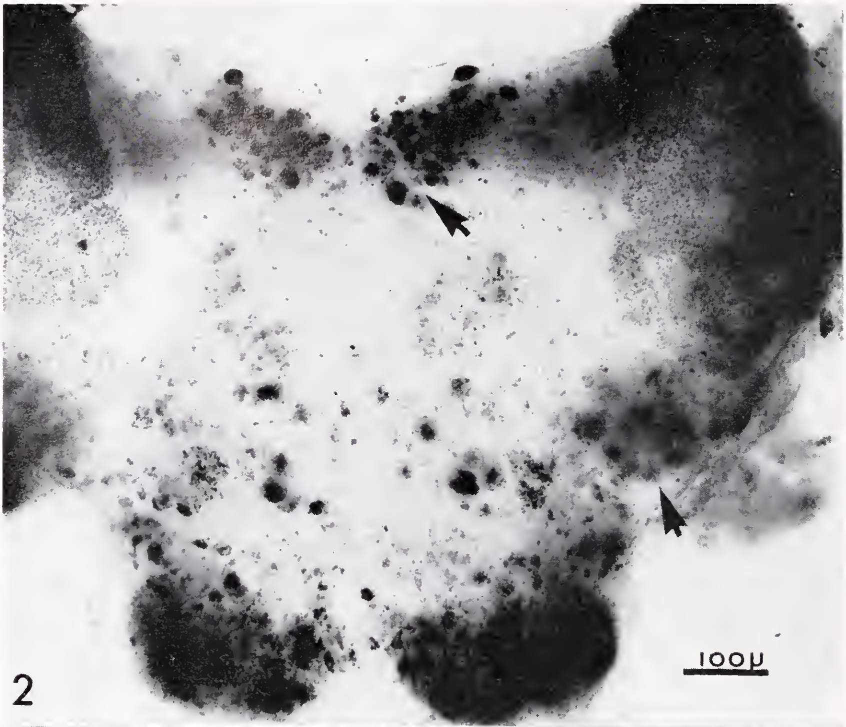
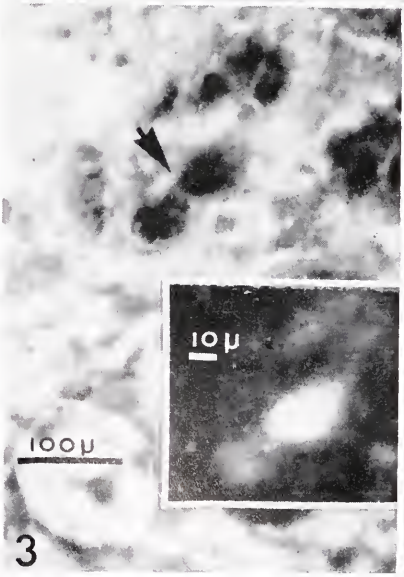


FIGURE 1. Locations of ganglionic nuclei in the cerebral ganglion of *N. virens* (adapted from Holmgren, 1916). a = antennal nerve; ao = anterior optic nerve; cc = circumoesophageal connective, serving cirri; nu = nuchal nerve; p = palpal nerve; po = posterior optic nerve.





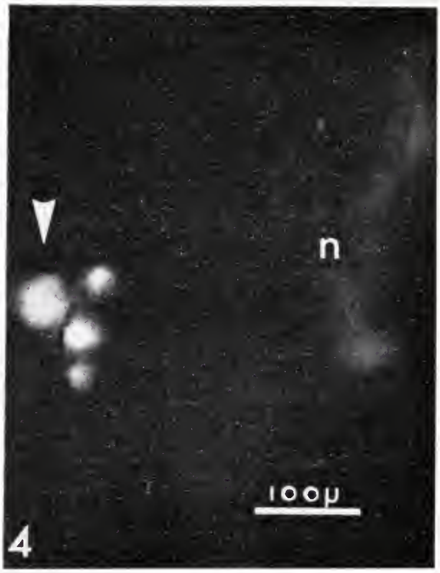


FIGURE 2. Whole mount of cerebral ganglion stained with neutral red. Dorsal view. Arrows indicate nuclei 4 and 14.

FIGURE 3. Nucleus 8: cryostat section, cresyl violet. Arrow indicates cyan fluorescing cells shown in insert.

FIGURE 4. Nucleus 14 showing large (arrow) yellow fluorescent cell adjacent to 3 small cyan fluorescent cells. N shows hazy fluorescence in neuropile.

the emission beam. Galvanometer deflections were recorded at 5 nm intervals. All spectra are expressed as relative quanta versus wave length.

Two tests for specificity of fluorescence were used. The sodium borohydride reduction test (Corrodi, Hillarp and Jonsson, 1964) and the quenching of fluorescence with water.

Cells exhibiting specific fluorescence were assigned to ganglionic nuclei largely by comparing their locations with those of the same cells in adjacent stained sections. Paraffin sections of the same area were also useful. The neuronal nuclei of the cerebral ganglion are numbered according to the system of Holmgren (1916) (Fig. 1). Although the number of fluorescing cells in various parts of each ganglion was recorded, it was decided that a numerical analysis would have little meaning since the exact distribution of fluorescing cells in any one ganglionic nucleus varied from one animal to another, possibly due to cyclic filling and discharge, and the precise localization of any one fluorescing cell in one specific ganglionic nucleus was not always possible.

RESULTS

A cluster of cells in each of two ganglionic nuclei, numbers 4 and 14, stained with neutral red in all cases. In addition a number of single cells stained with neutral red were always scattered over the dorsal side of the ganglion (Fig. 2).

Fluorescence microscopy

A single cerebral ganglion was estimated to contain an average of 45 to 50 cell bodies showing specific fluorescence, 15 to 20 of them yellow fluorescent and 30 to 35 cyan fluorescent. The fluorescence observed was confined to the cytoplasm and could not be followed any distance in the axons. Pin points of cyan fluorescence, probably representing sections of axons, were characteristic of the neuropile, where yellow fluorescence was rare and hazy (Fig. 4). This may be due to the relatively low fluorescent yield of indolylethylamines (Corrodi and Jonsson, 1967). Cyan fluorescent cells (Fig. 3) were present in all ganglionic nuclei exhibiting specific

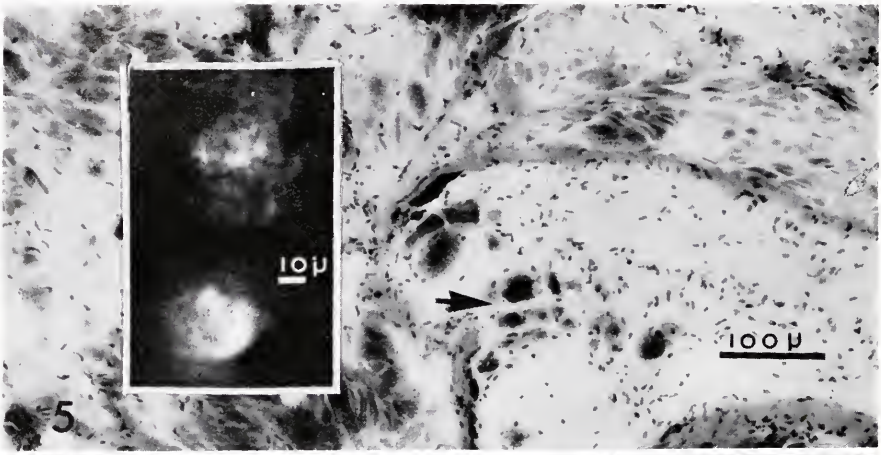
TABLE I

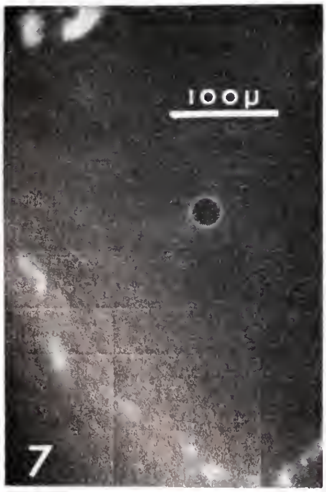
Distribution of biogenic amine fluorescence in the prostomium of Nereis virens.

Ganglionic nuclei	Fluorescence		Parts of nervous system	Fluorescence	
	Cyan	Yellow		Cyan	Yellow
4	+	+	Hindbrain	+	+
5	+	−			
6	+	−	Neuropile	Pin points	Haze → neg.
7	+	−	Antennal nerve	+	−
8	+++	+			
9	+++	+	Circumoesophageal connectives	+	−
10	+++	+			
11	+++	+	Cirral ganglion	+	−
14	+	+++	Cirral nerve	+	−
25	+	+	Dorsal epidermis	+	−

fluorescence (Table I), while the yellow fluorescence was more restricted in distribution, occurring most frequently in a few large (25–30 μ), bilaterally symmetrical cells in nucleus 14 (Fig. 4, Table I). Posterior to the neuropile there is a region, containing five nuclei, where one or two fluorescing cells were found in every animal. However, because of the complex structure of this part of the brain, it was impossible to assign these few fluorescent cells to particular nuclei with any degree of confidence.

Specific fluorescence was also characteristic of the prostomial nervous system peripheral to the cerebral ganglion (Table I). Cyan fluorescence was found in groups of cells (15–20 μ) along the circumoesophageal connective, including the





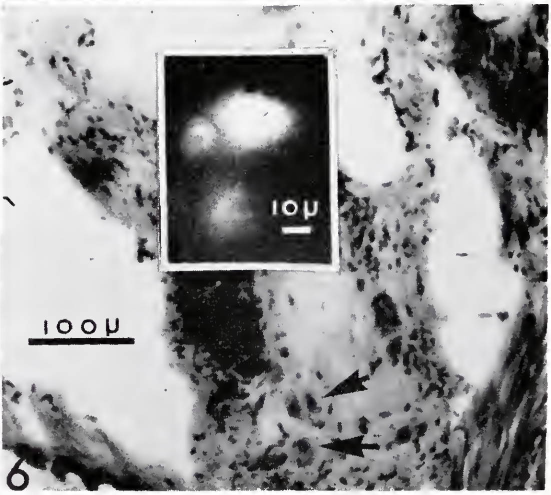


FIGURE 5. Neurons (arrow) in the circumoesophageal connective: cryostat section, cresyl violet. Arrow indicates fluorescing cells shown in insert.

FIGURE 6. Cirral ganglion: cryostat section, cresyl violet. Arrows indicate fluorescing cells shown in insert.

FIGURE 7. Fluorescent bodies around periphery of the base of the antennal nerve.

site of divergence of the cirral nerves, in small cells (10–15 μ) at the base of each cirrus (Fig. 6), faintly along the length of the cirral nerve and in a ring at the base of each antennal nerve (Fig. 7). No fluorescence was seen in this nerve distal to its base and none was found in the optic nerves, nuchal nerves, palpal nerves, tegumentary nerves or in the nerves serving the prostomial musculature. No yellow fluorescence was seen in any part of the prostomium outside the cerebral ganglion. Cyan fluorescent cells were also present in the dorsal prostomial epidermis, in cells which orient perpendicularly to the basement membrane directly above the base of the antennal nerves. They are similar in position and morphology to the fluorescent bipolar sensory cells described by Clark (1966) in *Nepthys* and are probably located among the epidermal sensory cells (Smith, 1957), which in nereids constitute a part of a peripheral sensory system continuous with the antennal nerves. Various forms of autofluorescence were seen in the prostomium of *N. virens* but, with the exception of one case described below, they did not interfere with spectral measurements.

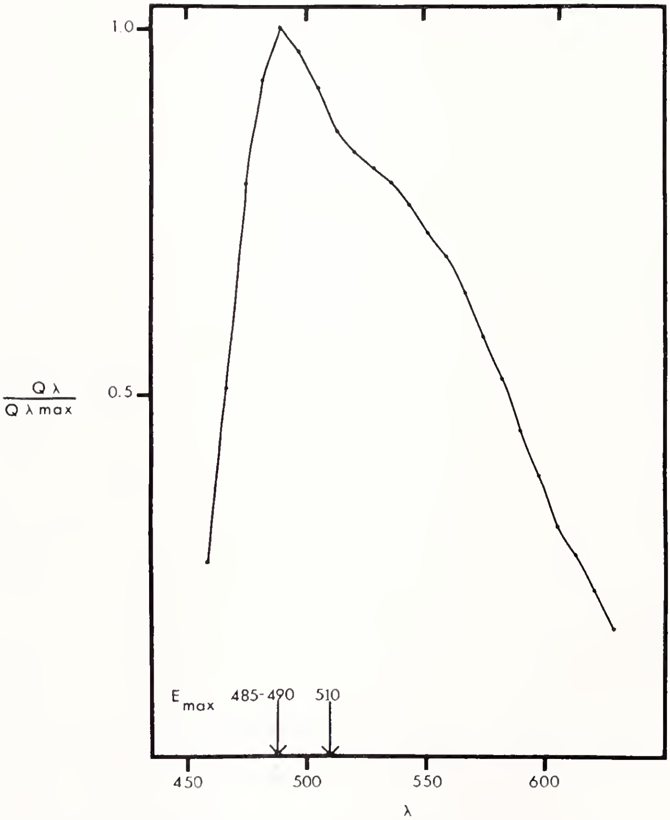


FIGURE 8. Emission spectrum of Type IY yellow specific fluorescence in cerebral neurons.

Microspectrofluorimetry

Yellow fluorescence. Emission spectra obtained from yellow fluorescing cells fall into two categories. Type IY spectra have a well defined maximum at 485 to 490 nm, but also a prominent shoulder in the falling phase, beginning at approximately 510 nm. (Fig. 8) which is presumably responsible for the yellow appearance of this fluorescence. Type 2Y spectra have a very broad emission maximum that extends from 485 to 535 nm (Fig. 9). The emission maxima of these spectra do not correspond with that for any single category of monoamine fluorescence as described by Björklund *et al.* (1971), although the emission maxima of these authors' Group B and Group C fluorophores all fall within the range of both Type IY and Type 2Y spectra. Type IY cells (26 measured) and Type 2Y cells (12 measured) could not be visually distinguished from one another by color and were not confined to separate ganglionic nuclei. In order to determine if the spectra were being distorted by photodecomposition of the fluorophore, readings on 24 cells

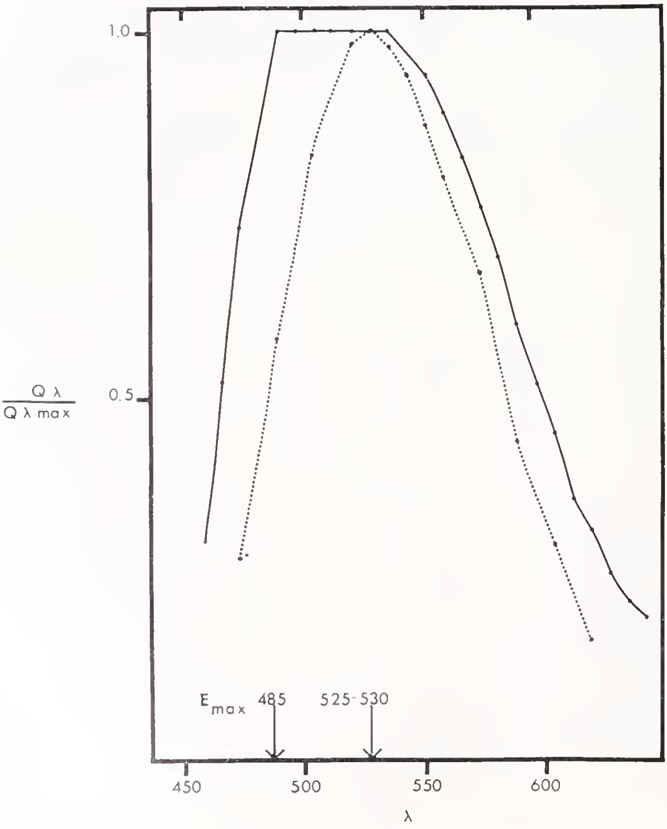


FIGURE 9. Emission spectra of Type 2Y yellow specific fluorescence in cerebral neurons (solid line) and of 5-HT (dotted line) in model droplets (0.002 M).

were taken as soon as the cell had been located and again after five or more minutes of irradiation. No significant alterations of the spectra were observed. The emission maximum for the model droplets of 5-HT was 525 to 530 nm (Fig. 9), which agrees with the maxima reported by others (Ritzén, 1966; Rude, Coggeshall and Van Orden, 1969; Van Orden, 1970; Björklund *et al.,* 1971 and Björklund, Falck and Omen, 1972). Since the maximum of the Type 2Y spectrum extends past the maximum of 5-HT, it is possible that these cells contain 5-HT, although the presence of other formaldehyde-induced compounds excited at 410 nm seems likely. The shoulder of the Type 1Y spectrum approximates the emission maximum for 5-HT, so it is also possible that small amounts of this material occur in the Type 1Y cells. There are a number of indolylethylamines, 5,6-dihydroxytryptamine, 6-hydroxytryptamine, α-methyl-5-hydroxytryptamine and 5-hydroxytryptophan (Jonsson and Sandler, 1969; Björklund *et al.,* 1971) with fluorescence yields comparable to or greater than that for 5-HT and with maxima that fall within the emission maximum of the Type 2Y spectrum. It is possible, therefore, that one or more of these indolylethylamines is contributing to both types of yellow specific fluorescence seen in *N. virens.* In addition, the emission maximum of the Type 1Y cell falls within the maximum (490–500 nm) of the tryptamine fluorophore.

When exposed to UV light indolylethylamine fluorophores decompose more rapidly, except for 6-HT (Jonsson and Sandler, 1969), than do catecholamine fluorophores. Although absolute photodecomposition rates are not available, Casperson, Hillarp and Ritzén (1966) reported that the 5-HT fluorophore fades twice as rapidly as does a catecholamine fluorophore, a relationship which also applies to our yellow *vs.* cyan fluorescence and 5-HT *vs.* noradrenalin standards. These results also imply that 6-HT, which has a high fluorescent yield and fades slowly, is not a major component of either type of yellow fluorescence. However, we found that the photodecomposition rate of the 5-HT standard was distinctly more rapid than that of the yellow cells, due possibly to the fact that the most rapid photodecomposition of the fluorophore of any biogenic amine occurs during the first three minutes of irradiation (Ritzén 1966), so that by the time the cell had been located, the rate of photodecomposition had passed its peak. Alternatively, the fluorophore in the yellow cell body may have an innately slower rate of photodecomposition than does 5-HT. Most fluorophores of indolylethylamines have similar fading characteristics (Jonsson and Sandler, 1969) so that this measure does not serve to differentiate 5-HT from the others. Another possibility, that a shift in catecholamine emission maximum from about 490 to 540 nm, known to occur at high concentrations (Corrodi and Jonsson, 1967), might be contributing to the Type 1Y and 2Y spectra, was investigated by subjecting the sections to a less humid paraformaldehyde and a shorter incubation time, a procedure known to prevent such a change in emission maximum (Jonsson, 1971). With this treatment, the specific fluorescence in the yellow cells was less intense but their spectra had not changed and, in addition, the characteristic emission spectra of adjacent cyan fluorescing cells remained unaltered. The emission spectrum of the autofluorescent glial cells in the cerebral ganglion reaches a maximum at 485 to 490 nm, and extends as a plateau to approximately 565 nm. Spectral readings to determine whether or not glial cells adhering to fluorescent neurons might be contributing to the emission spectra of the specific fluorescence were attempted, but the fluorescent intensity of the glia

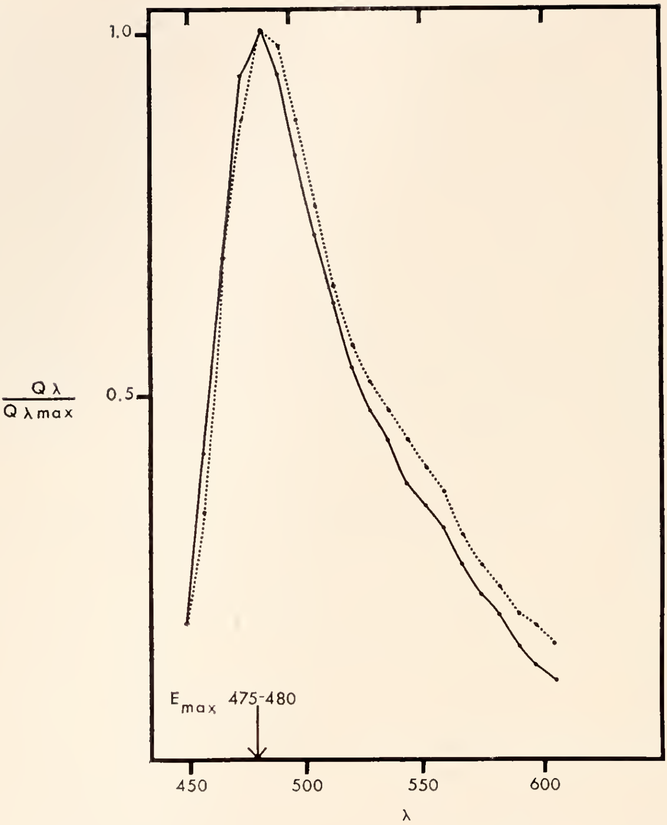


FIGURE 10. Emission spectra of cyan specific fluorescence in cerebral neurons (solid line) and for noradrenalin (dotted line) in model droplets (0.002 M).

was so low that it could not be read by the photomultiplier tube at the amplifications employed for measurement of specific neuronal fluorescence.

Cyan fluorescence. The cyan fluorescing cells and nerve fibers in the cerebral ganglion, circumoesophageal connectives, prostomial nerves and other ganglia of *N. virens* all display an emission spectrum with a well defined maximum at 475 to 480 nm, (Fig. 10), characteristic of the catecholamines dopamine, noradrenalin and adrenalin (Ritzén, 1966; Björklund *et al.*, 1972). However, adrenalin, a secondary catecholamine, requires more energy during dehydrogenation with formaldehyde due to the formation of a quaternary nitrogen in the indole nucleus (Jonsson, 1967), and a longer incubation time may, therefore, be required to bring about the formation of the adrenalin fluorophore (Corrodi and Jonsson, 1967). *N. virens* tissue sections were incubated for an additional 2 hours, but this procedure did not result in new fluorescing structures or in any increase in the intensity of fluorescence. The photodecomposition rate for the cyan fluorescence corresponded more closely to that for the noradrenalin standard than did the yellow fluorescence to the 5-HT standard, perhaps because catecholamine fluorescence is more resistant to photodecomposition than is indolylethylamine fluorescence. The cyan fluorescence of the epidermal cells was not recorded successfully because it tended to be diffuse and was effectively masked by the autofluorescence (E_{max} 480–490 nm) of the adjacent cuticle.

Tests for specificity. The application of the first step of the sodium borohydride reduction test, the immersion of cryostat sections in 100% ethanol, resulted in the disappearance of all fluorescence. Consequently there was no point in pursuing the second step, the incubation of sections in a solution of $NaBH_4$. The biogenic amines of *N. virens* may be bound only lightly to tissue proteins and may, therefore, be extracted easily by organic solvents. The fluorescence of *N. virens* tissue faded and disappeared when water was added, and after 15 minutes specific fluorescence could not be distinguished from background autofluorescence. Ritzén (1966) reported similar results with noradrenalin and 5-HT fluorophores. When these wet tissue sections were subsequently dehydrated the fluorescence did not reappear.

DISCUSSION

The yellow fluorescent neurons described in this study are similar in distribution to those found in the cerebral ganglion of *Nereis diversicolor* and *N. pelagica* by Warembourg and Dhainaut-Courtois (1969) and Dhainaut-Courtois (1972). This paper, however, provides the first evidence for cyan fluorescent cells in the nereid brain, as well as the first account of the spectral characteristics of yellow and cyan specific fluorescence in a polychaete brain.

The spectral characteristics and photodecomposition rates of the yellow fluorescence, Types 1Y and 2Y, indicate that at least one indolylethylamine, (and probably more), is present. There are, however, at least five known indolylethylamines, including 5-HT, with emission spectra and photodecomposition rates that fall within the parameters of our fluorophores (Jonsson and Sandler, 1969). One particularly likely candidate is tryptamine (E_{max} 490–500 nm), which fits fairly well with the peak of the Type 1Y spectrum. Tryptamine is a precursor of all indolylethylamine derivatives and it is possible that the 1Y and 2Y spectra reflect two distinct metabolic conditions in the yellow neurons, with tryptamine in particularly high concentrations in Type I. Since all 'yellow' neurons fall clearly into either Type I or Type II categories, they presumably represent either two phases of one sort of neuron or two distinct kinds of neuron. Bufotenine, suggested by Dhainaut-Courtois, Caridroit and Bizerte (1969) as the source of a specific yellow fluorescence with an emission maximum (no spectral curve published) at 525 nm in the nervous systems of *N. diversicolor* and *N. pelagica,* cannot be seriously considered, since this substance is a tertiary amine and should not, therefore, fluoresce (Corrodi and Jonsson, 1967; Björklund *et al.*, 1971). Yellow fluorescing cells with a broad emission maximum have also been reported for *Hirudo* (Ehinger, Falck and Myhrberg, 1968), *Lumbricus* (Ehinger and Myhrberg, 1971) and the regenerating nervous system of *Allolobophora* (Koritsánszky and Hartwig, 1974). These few microspectrofluorimetric studies present a situation similar to that found in *N. virens* and suggest that the spectral characteristics of the yellow fluorescence in annelid nervous sysems are likely to be complex and may involve a number of indolylethylamines.

The Retzius cells of the leech, on the other hand, appear to contain 5-HT only (Rude *et al.,* 1969), and biochemical analysis of the ventral nerve cord of *Arenicola marina, Amphitrite ornata, Glycera dibranchiata* (Welsh and Moorehead, 1960) and *Glycera convoluta* (Manaranche and l'Hermite, 1973) have shown the presence of significant amounts of 5-HT. It seems likely, therefore, that this amine may be one component of the yellow specific fluorescence seen in *Nereis.*

We have several clues as to the function of indolylethylamines in annelids. Recently Marsden and Lacalli (1978) have shown that 5-HT appears to stimulate mucus release by larvae of *Arenicola cristata.* Lent (1973) argued that the Retzius cells control mucus secretion by the skin of the leech, and has suggested (1977) that mediation of secretion may be an evolutionarily ancient rôle for 5-HT. Coleman (1975) disputes Lent's position and considers it more probable that the 5-HT in the Retzius cell serves to modulate the action of other, probably cholinergic, neurons exciting the mucous cell A related argument has been proposed by Alvarez, del Castillo and Sanchez (1969), who found that 5-HT appeared to modify the excitatory effect of ACh on longitudinal body wall muscle in the sabellid *Sabellastarte.* There may, perhaps, be some phylogenetic significance to the fact that large, yellow fluorescing neurons are characteristic of the ventro-lateral nucleus 14 in *Nereis diversicolor, N. pelagica* (Warembourg and Dhainaut-Courtois, 1969), *N. virens,* and the ventro-lateral region of the cerebral ganglion of the related genus *Nephtys* (Clark, 1966). In *N. virens* there are also smaller numbers of such cells on the dorso-medial nuclei 4, 10, and 11. All these cell groups lie close to the roots of bundles of nerve axons passing to prostomial palps, tentacles and cirri richly supplied with mucous cells. The absence of peripheral yellow fluorescence, which appears to argue against indolylethylaminergic innervation of mucous glands, may, as mentioned earlier, be an artifact caused by rapid fading. Peripheral destinations for processes from central 'yellow' cells, although undemonstrated in *Nereis,* may exist. Yellow specific fluorescence has been seen in the antennal nerves of both *Nephtys caeca* (Clark, 1966) and *Glycera convoluta* (Manaranche and l'Hermite, 1973). Also, since the cerebral ganglion of *Nereis* is almost certainly involved in habituation and learning (Evans, 1969; Evans *et al.,* 1974) and neurosecretion, (Clark and Olive, 1973) an interneuronal role of some kind for indolylethylaminergic neurons within the cerebral ganglion is a reasonable possibility, although one we are far from being able to substantiate.

The spectral characteristics of the cyan fluorescence found in the cerebral ganglion and prostomial nerves of *Nereis virens* indicate that a catecholamine is present. Although biochemical and fluorescence histochemical studies on other nereids (*N. pelagica* and *N. diversicolor*) have failed to demonstrate the presence of catecholamines (Warembourg and Dhainaut-Courtois, 1969; Dhainaut-Courtois, 1969; 1972), our discovery of catecholamines in *N. virens* is in accord with biochemical studies on *Glycera* (Manaranche and l'Hermite, 1973), bioassays on *Arenicola* (Östlund, 1954), as well as with microspectrofluorimetric measurements for *Lumbricus* (Ehinger and Myhrberg, 1971), *Allolobophora* (Koritsánszky and Hartwig, 1974) and *Hirudo* (Ehinger *et al.*, 1968). Since all catecholamines and their α-methylated derivatives and corresponding amino acids (DOPA and -m-DOPA) have the same spectral characteristics (Jonsson, 1967), it is not possible, from our evidence, to say which substance(s) is present. However, our analysis indicates that adrenalin is unlikely. The biochemical studies on *Arenicola marina* (Östlund, 1954) and *Glycera convoluta* (Manaranche and l'Hermite, 1973) suggest that noradrenalin and dopamine are possibilities.

Although there is no experimental evidence for a functional rôle for catecholamines in annelids (Gerschenfeld, 1973), the cyan fluorescence in prostomial epidermal cells along the cirral and at the base of the antennal nerves implies catecholaminergic pathways which correspond morphologically with an epidermal sensory system characteristic of nereids (Smith, 1957). Catecholaminergic cells, interpreted as sensory, have also been observed in the prostomial integument in *Lumbricus* (Dahl, Falck, von Mecklenburg and Myhrberg, 1963) and *Nephtys* (Clark, 1966). Within the cerebral ganglion, although there are probably some sensory cells, such as those in nucleus 18 (Whittle and Zahid, 1974), it seems unlikely that the rather substantial numbers of catecholaminergic neurons, most of them not in the vicinity of nucleus 18, are all sensory. It is more probable, particularly in view of the abundance of cyan fluorescence in the neuropile, that most of the cerebral catecholaminergic cells serve some collecting, controlling or modifying purpose. In this connection the presence of aminergic neurons in the hindbrain, in close proximity to probable neuro-secretory cells, is interesting and deserves further study. Therefore, although some of our findings are compatible with the fairly widely considered hypothesis (Dahl *et al;* 1963; Clark, 1966; Myhrberg, 1967; Marsden and Kerkut, 1969 and Welsh. 1973) that in annelids catecholaminergic neurons are sensory and indolylethylamine-containing cells are motor, it seems likely that at least some of the aminergic neurons located in the cerebral ganglion of *N. virens* belong to the third category of interneurons and function in a variety of different ways.

This research was supported by an Operating Grant from the National Research Council of Canada to Joan Marsden and made possible by the technical expertise of Robert Lamarche, whose help is gratefully acknowledged.

SUMMARY

1. Vital staining with neutral red reveals that possible biogenic amine-containing cells are abundant in the cerebral ganglion of the polychaete, *Nereis virens.*

2. Fluorescence microscopy indicates two kinds of cells exhibiting specific fluorescence, one blue-green (cyan) and the other yellow.

3. The emission spectrum recorded from the cyan fluorescing cells is characteristic of a catecholamine, which is probably not adrenalin.

4. Emission spectra recorded from yellow fluorescing cells are of two types, both suggestive of a mixture of indolylethylamines. It is probable that 5-HT is one component of these spectra. A possible relationship between some of these yellow cells and prostomial mucous glands is discussed.

5. Yellow specific fluorescence was confined to the cerebral ganglion where cyan fluorescence was also seen in peripheral areas, a distribution suggestive of a sensory rôle for peripheral catecholaminergic cells.

6. The probability that both catecholaminergic and indolylethylamine-containing neurons in the cerebral ganglion serve to control or modify other neural or neurosecretory activity is discussed.

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MONTHLY REPRODUCTIVE CYCLES IN THREE SYMPATRIC HOOD-BUILDING TROPICAL FIDDLER CRABS (GENUS *UCA*)

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Since the early studies of Korringa (1947), several intertidal invertebrates have been reported to exhibit semi-monthly cycles of reproductive activity (see Neumann, 1975; Klapow, 1976). Included in these studies are several utilizing fiddler crabs (genus *Uca*) (Crane, 1958; Feest, 1969; von Hagen, 1970; Zucker, 1976; Christy, 1978). This paper confirms and quantifies the reported cyclicity of reproductive activity in *Uca* but reveals a monthly (rather than semi-monthly) cycle tied to a specific lunar phase in three tropical *Uca* species. Male courtship activity reached significant peaks during the spring tides occurring after the Full Moon (FM).

Surface activity (which includes courtship, feeding, and combat) for these intertidal crabs is restricted to low-tide periods. Temperate species hibernate in the winter (Crane, 1943), but males court (*i.e.,* wave their major cheliped) throughout all low-tide periods in the warmer months (Salmon, 1965). Subtropical species, exposed to longer periods of warm weather, court only during diurnal and early evening low-tide hours (Salmon and Atsaides, 1968). Tropical species court in the daytime throughout the year but have only rarely been reported to court at night (Crane, 1975). Many tropical species do not even leave their burrows during nocturnal low tides (personal observation). Thus, there is an inverse relationship between the number of warm months in a given region, and the amount of time devoted to courtship activities on a daily basis.

Observations by Crane (1958) in Trinidad on *U. maracoani,* by Feest (1969) on *U. annulipes* and *U. triangularis* in South India, by von Hagen (1970) on four Trinidad species, by Christy (1978) on *U. pugilator* in Florida, and my own earlier work on *U. musica terpsichores* in Panama (Zucker, 1973, 1976) suggested a further reduction in the daily hours of courtship in tropical forms. Courtship in *U. maracoani* was restricted to those low-tide periods coinciding with early morning hours (Crane, 1958). In *U. annulipes* and *U. triangularis,* waving peaked between 1000 to 1100 hr (Feest, 1969). Four of the seven *Uca* species found on Trinidad were observed by von Hagen (1970) to restrict courtship activities to low tides occurring around the Full Moon (FM) and New Moon (NM) (late morning hours). Christy (1978) found courtship most prevalent in *U. pugilator* during the week following each Quarter Moon. In *U. m. terpsichores* courtship occurred during late morning through mid-afternoon low-tide periods, *i.e.,* during FM and NM spring tides (Zucker, 1973, 1976). Other diurnal low-tide periods were devoted to feeding, burrow repair, aggression, wandering or remaining within the burrow. Since the low-tide period occurs approximately 50 min later each day, about 15 days pass before low tide again coincides with the observed hours of courtship activity. Hence, it appears from these studies that male *Uca* were exhibiting semi-monthly cycles of courtship and other activities and were perhaps synchronizing their daily behavior patterns with one another (Crane, 1958; Barnwell, 1968; Zucker, 1976). Recent evidence suggests that females of some *Uca* species are reproductively receptive mainly around the FM period (von Hagen, 1962; Zucker, 1973) while other species exhibit peaks in receptivity twice each month (von Hagen, 1970; Christy, 1978).

Although all previous studies revealed a semi-monthly rhythm of male courtship activity, these studies were, for the most part, either nonquantitative or dealt with small sample sizes. Crane's (1958, 1975) most extensive work in this area involved six male *U. maracoani* in an artificial outdoor "crabbery" in Trinidad. To confirm and extend Crane's observations, the amount of time individually-marked male *U. m. terpsichores* spent performing various behaviors on a daily basis was recorded in the field starting 1 hr past dead low tide (Zucker, 1973, 1976). This study, too, suffered from a small sample size (only five males and no females were monitored each day). Feest (1969) merely described general impressions of when courtship peaked in her study populations, and von Hagen (1970) indicated days in which more or less than 50% of the males were courting.

The present work utilized the three sympatric Panamanian hood-building (formerly, shelter-building) species as part of a larger investigation of the social organization of these crabs. This paper examines the degree of synchronization of behaviors within these species in order to ascertain the timing of courtship and other activities within the lunar month in a quantitative manner.

Crane (1941) originally used the term "shelter" to describe the semi-domes of mud some males constructed over the mouth of their burrows. In her recent major work, Crane (1975) has adopted the term "hood" which was previously used by Matthews (1930). The term "hood" is more neutral in connotation than "shelter" which implies a function for these structures. Although some previous workers assumed they functioned to protect or shelter the crabs from the sun (Matthews, 1930), recent work has shown that they function in a social context. Zucker (1974; and in preparation) has shown that the presence of hoods serves to reduce the size of a male's territory since he only courts in front of it rather than in a 360° circle around his burrow as non-hood owners do. This reduction in courtship area lowers the amount of overlap between neighboring territories. Aggressive encounters are likewise reduced (Zucker, in preparation). Von Hagen (1968, 1972) suggested hoods might attract females to the males' burrows. This possibility will be tested in the future.

Materials and Methods

Observations were made from July 19 to August 14, 1975, on an intertidal muddy-sand flat in the Panama Canal Zone. The flat, along the east bank of the Pacific entrance to the Canal, is the same one described by Crane (1941) as housing 15 species of *Uca*. The upper flat was less muddy than the remainder. This muddy-sand area was inhabited mainly by the three Panamanian "hood-builders," *U. musica terpsichores, U. latimanus* and *U. beebei*.

Three "permanent" transects were placed in the muddy-sand region, each of which contained predominantly one of the three species. Each transect was marked by sinking four 1.52 m × 1.27 cm steel rods into the ground about 1.45 m. Nylon cord was tied around these posts during observations each day. The *U. m. terpsichores* transect was uppermost on the flat and measured 66 cm × 625 cm (4.13 m^2). The *U. latimanus* transect, located 5.40 m below and 15.66 m to the west side of the *U. m. terpsichores* one, was originally 75 cm × 658 cm (4.94 m^2). It was changed to 70 cm × 542 cm (3.79 m^2) after the rods were dislodged by wave action on July 21. The *U. beebei* transect, located 3.6 m below the *U. latimanus* one, was initially 75 cm × 625 cm (4.69 m^2). It was enlarged to 145 cm × 678 cm (9.83 m^2) after these rods were also dislodged on July 21. The *U. beebei* transect was made considerably larger than the others in order to compensate for the lower density of males in that population (see Fig. 1). No other rod dislodgements occurred during the course of the study. Observations were made 3 m from each transect.

Each day, starting at low tide and again at one hour past dead low tide, the "instantaneous" behavior of each individual within the three transects was recorded (see Altmann, 1974). These hours included the height of courtship activity. Males perform a daily sequence of behavior patterns starting with emergence from their burrows several hours before dead low tide. If courtship activity occurs during a given low-tide period, it will not be evident until about the hour of dead low tide. Each transect was scanned with the aid of binoculars. A verbal description of the species, sex, possession of a hood, and behavior of each individual at the moment of observation was recorded on a tape recorder for later transcription. The behaviors recorded included: feeding, waving (the major cheliped), waving-and-feeding (simultaneous waving motion by major cheliped and feeding motion by minor cheliped), combat, staying "in burrow" (recorded when a hood but no crab was present or a crab was seen leaving its burrow during the scan), digging, wandering, cleaning (sand off crab's carapace) and standing still. Each transect scan took from 2 to 10 min depending upon the number of crabs active, the behaviors being performed and the number of disturbances from predators. The order in which the transects were scanned was randomized from day to day.

The data were organized according to the hour of low tide rather than on a daily basis. This method was chosen to facilitate comparisons with past and/or future work. During several hourly intervals (0900–0959 hr, 1100–1159 hr after the FM, and 1100–1159 hr after the NM) the tidal advance slowed enough so that low tide on two consecutive days occurred during the same hourly interval. When this happened, the data were averaged for the two days. The results from the two hourly scans are fairly similar. The one hour past low-tide set of data is reported here in order to compare it to earlier work (Zucker, 1973, 1976) which was also based on observations made one hour past low tide.

RESULTS

The results of the scan samples reveal a concentration of courtship activities during the days just after the FM for all three species.

Figure 1 shows the observed density of active males and females, respectively, for each species at one hour after low tide for each low-tide hour. For example,

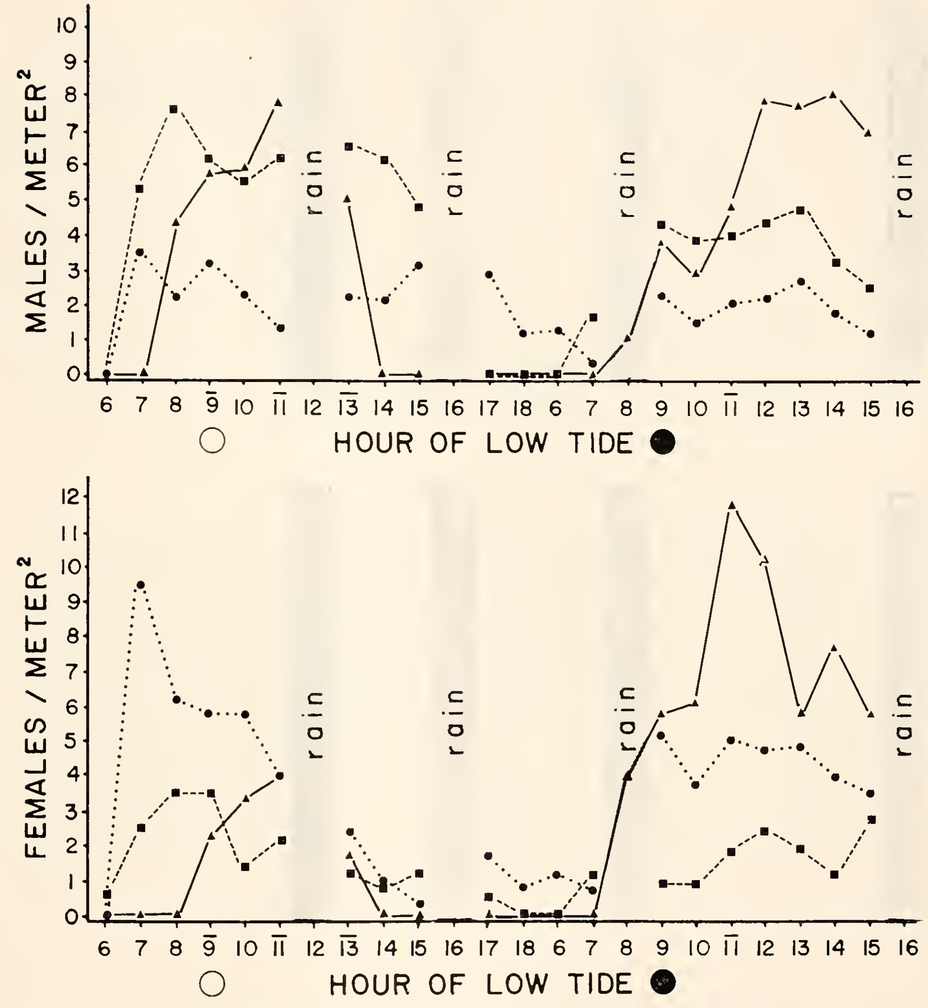
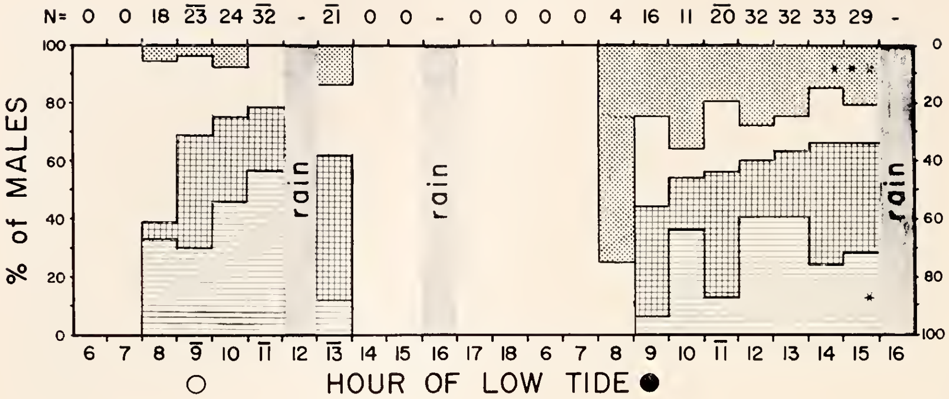


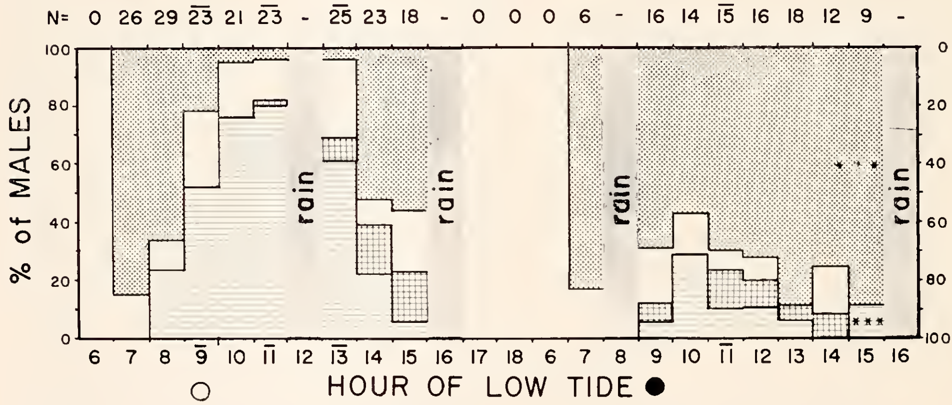
FIGURE 1. The density of male and female *Uca musica terpsichores* (triangles), *U. latimanus* (squares), and *U. beebei* (circles) observed on the surface of their respective transects, as a function of low tide hours. Hours in which two consecutive low tides occurred during that hourly interval are represented by an "average" bar over the hour on the abscissa. Data were gathered from July 19 to August 14.

low tide on July 24 occurred between 1000 hr and 1059 hr (represented as 10). The actual observations on that day were made about 1 hour later. The precise density of crabs in each transect could not be determined since some individuals may have been temporarily within their burrows as the transect was scanned. Male *U. m. terpsichores* densities reached peaks several days after both the FM and NM. Male *U. latimanus* density was about the same as *U. m terpsichores* during the FM period but only about half that many emerged during the NM

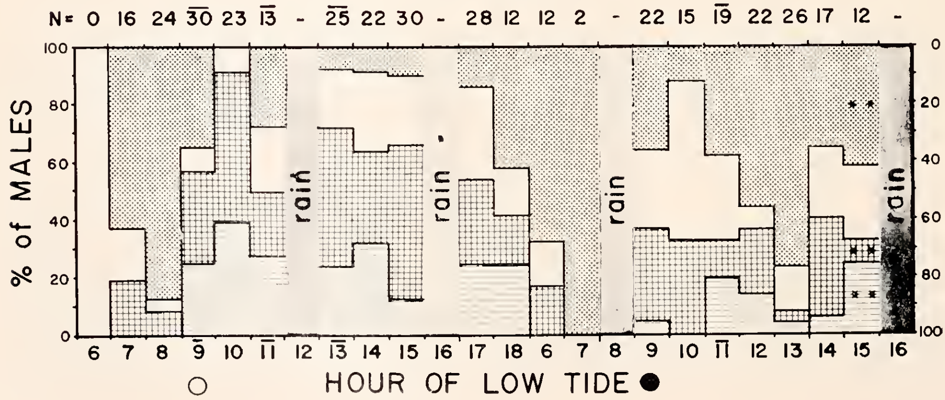
UCA MUSICA TERPSICHORES



UCA LATIMANUS



UCA BEEBEI



period. Male *U. beebei* density was at most only about half that of the other two species during their peaks. However, *U. beebei* density dropped to zero only during the early morning low-tide hours, while *U. m. terpsichores* and *U. latimanus* males stopped emerging in the late afternoon period as well (Fig. 1). Females of all three species were rarely observed from several days after the FM to just before the NM. Most *U. latimanus* and *U. beebei* females were observed on days just prior to the FM; *U. m. terpsichores* females were seen mainly after the NM (Fig. 1).

The days on which population densities dropped to zero parallel fairly closely the days on which the previous high tide did not cover the particular transect. Of the three transects, only the *U. beebei* transect was low enough in the intertidal zone to be covered by each high tide. The *U. latimanus* transect was covered by high tide from 0800 to 1600 hr during the FM period and from 0700 hr onwards during the NM period. Thus, there were five days in which high tide did not reach this transect. The highest transect (*U. m. terpsichores*) was covered by high tide from 0900 to 1200 hr around the FM and from 0800 hr onward around the NM. Thus, this transect was exposed at high tide during 11 of the 27 days of the study.

The percentage of male *U. m. terpsichores*, *U. latimanus* and *U. beebei* performing each of three main activities (feeding, waving, or waving-and-feeding) relative to the number of active conspecific males each hour of low tide is shown in Figure 2. The blank area on each graph represents all other activities: mainly those male crabs seen just emerging from their burrows (recorded as "in burrow") as well as a small amount of combat, cleaning, digging, standing still or wandering. Courtship (waving, and waving-and-feeding) peaked during the spring tides several days after the FM in all three species. Only in *U. m. terpsichores* was courtship also fairly prominent after the NM. Feeding was more prominent during the week surrounding the NM in all three species (Fig. 2). A chi-square test was used to compare the frequency of each of the three main behaviors and all other behaviors during the FM *vs.* NM weeks for each species (Table I). For all three species, significantly more males were waving, while significantly fewer males were feeding, around the FM than the NM ($P < 0.05$). Only in *U. beebei* were there significant differences from one week to the other for waving-and-feeding and for all other activities (other).

U. latimanus males exhibited the strongest population synchrony of activities (Fig. 2). For example, fully 80% of the active males were waving during the 1100 hr low tide period following FM (includes two consecutive days) (Fig. 2). Nearly 60% or more of the *U. latimanus* fed each day during the week following the NM (Fig. 2). Waving-and-feeding was more prominent in *U. m. terpsichores* and *U. beebei* than in *U. latimanus*. If all courtship activities (waving and waving-and-

FIGURE 2. Percentage of male *Uca musica terpsichores*, *U. latimanus* and *U. beebei*, respectively, performing various behaviors during each low tide hour. An "average" bar over a low-tide hour represents hours in which two consecutive low tides occurred during that hourly interval. Dotted area is percentage of males feeding, lined area is percentage waving, cross-hatched area is percentage waving-and-feeding and blank area is percentage in burrow or performing other infrequent activities. The asterisks on the right indicate significant differences in the number of males performing each behavior during the FM period *vs.* the NM period ($* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$).

TABLE I

The number of male Uca m. terpsichores, U. latimanus *and* U. beebei *in each activity during the weeks of the full moon (FM) and new moon (NM) (low tides from 0600–1100 hr and 1300–1500 hr). No data were collected for FM 1200 hr, or after 1500 hr following the NM. Therefore, the corresponding data from the other moon phase period were deleted from the test.*

Behavior	*U. m. terpsichores*			*U. latimanus*			*U. beebei*		
	FM	NM	*P**	FM	NM	*P*	FM	NM	*P*
Feeding	7	34	<0.001	71	68	<0.001	53	58	<0.01
Waving/feeding	34	49	>0.50	10	5	>0.90	65	25	<0.01
Waving	45	36	<0.05	74	8	<0.001	37	8	<0.01
Other**	32	26	>0.10	33	9	>0.10	28	34	<0.02
Total	118	145		188	90		183	125	

* A two-tailed chi-square test was used to compare NM with FM period for each behavior.
** Includes: "in burrow," combat, digging, cleaning carapace, standing still, and wandering.

feeding) are pooled for *U. m. terpsichores* and *U. beebei,* strong population synchrony of courtship is also seen in these species during the FM period (Fig. 2).

The frequency of hood-building by each of the three species is shown in Figure 3. Only about 15% of all *Uca* species have been observed to build these structures (Crane, 1975). Of the 29 Panamanian species only the three reported here display this behavior. All three species restricted building mainly to a few days following the FM. A few hoods were also constructed after the NM. *U. latimanus* males again displayed the greatest synchrony. During peak construction days 83 to 100% of the *U. latimanus* males built hoods.

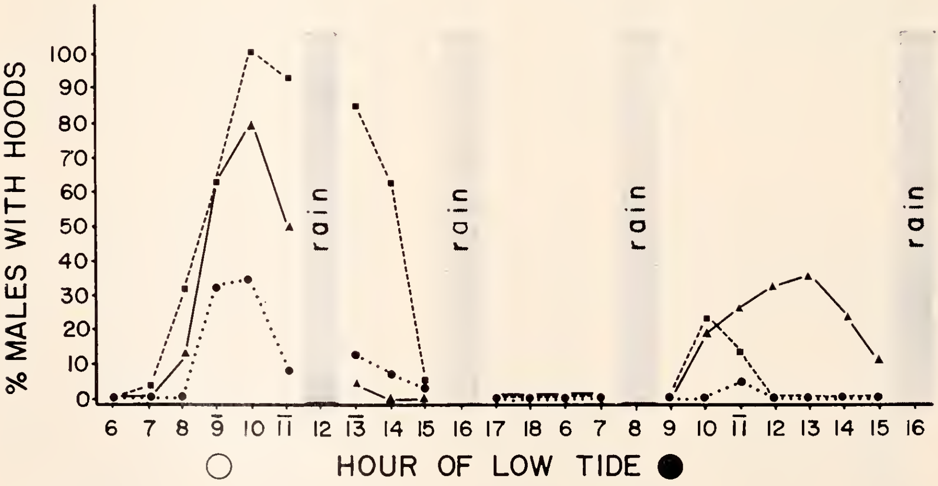


FIGURE 3. Percentage of male *Uca musica terpsichores* (triangles), *U. latimanus* (squares), and *U. beebei* (circles) building hoods each low tide hour. An "average" bar over a low-tide hour represents hours in which two consecutive low tides occurred during that hourly interval.

TABLE II

A composite of the various behavior patterns performed by males of the three Uca *species from July 19 to August 14, 1975 (low tides from 0600–1800 hr; 0600–1500 hr). All observations were made approximately one hour after low tide.*

Behavior of ♂♂	*U. m. terpsichores* N = 368 (%)	*U. latimanus* N = 380 (%)	*U. beebei* N = 457 (%)
Feeding	57 (15.49)	167 (43.95)	155 (33.92)
Waving/feeding (W/F)	114 (30.98)	20 (5.26)	131 (28.67)
Waving	117 (31.79)	129 (33.95)	77 (16.85)
Threat & combat	30 (8.15)	8 (2.11)	21 (4.60)
Other*	50 (13.59)	56 (14.74)	73 (15.97)
All feeding activities (W/F and feeding)	171 (46.47)	187 (49.21)	286 (62.58)
All courtship activities (W/F and waving)	231 (62.77)	149 (39.21)	208 (45.51)
Hood-building	111 (30.16)	163 (42.90)	35 (7.66)

* Includes: "in burrow," digging, wandering, cleaning carapace, and standing still.

Table II is a composite of the number of males performing each activity for the entire observation period from July 19 to August 14. Over the entire month's observations, more than 350 males were observed for each species. The percentage of males performing each behavior varied between species. Compared to the other two species, less than half as many *U. m. terpsichores* males fed at this time each day over the entire period. The number of males engaged in waving was about the same for *U. m. terpsichores* (32%) and *U. latimanus* (34%); only half as many *U. beebei* engaged in waving (17%). However, fewer *U. latimanus* males were engaged in all courtship activities (waving and waving-and-feeding) than the other two species. Combat was rarely seen in all three species: 8% for *U. m. terpsichores;* 2% for *U. latimanus;* and 5% for *U. beebei.* The percentage of *U. latimanus* males building hoods was higher than their percentage of waving males. This occurred since some of the hood-builders were in their burrows either briefly or perhaps with a female while the scan was being taken. Almost as many *U. m. terpsichores* built hoods as waved; only half as many *U. beebei* males constructed

TABLE III

Correlations between the frequency of each of the three main behavior patterns with hood-building (N is the number of days on which males were active.)

	U. m. terpsichores N = 16		*U. latimanus* N = 19		*U. beebei* N = 21	
	r	*P**	r	*P*	r	*P*
Hood-building *vs.*						
Feeding	−0.56	<0.05	−0.93	≪0.001	−0.45	<0.05
Wave/feed	−0.06	≫0.10	−0.16	≫0.10	0.37	>0.10
Waving	0.74	<0.01	0.95	≪0.001	0.55	<0.05

* Two-tailed test (Diem and Lentner, 1970).

hoods as waved. The relationship between feeding or courtship and hood-building for all three species is clearly seen from the correlation coefficients (Table III). In all three species there is a significant positive correlation between the number of males waving and the number of hoods constructed as well as a significant negative correlation between feeding and construction. *U. latimanus* exhibits the strongest correlations, while *U. beebei* exhibits the weakest.

A graph showing female behavioral activity is not included since most of their activities are difficult to monitor by the scan sample technique. Except for feeding, female activities take place away from the burrow entrance. These behaviors include mostly wandering (a presumed indicator of female sexual receptivity; Crane, 1975) and remaining in her own burrow, or in a male's burrow (presumably for mating). Of all the females observed, 94%, 92%, and 95% of *U. m. terpsichores* (N = 350), *U. latimanus* (N = 148), and *U. beebei* (N = 799), respectively, were seen feeding during the daily scans. One female *U. m. terpsichores,* three *U. latimanus* and four *U. beebei* were seen wandering. All but one of these eight females wandered between 1000 to 1300 hr after either the NM (two females) or FM (five females). Five matings were observed on the surface during the *U. beebei* scans. They occurred at the following times: two at 1300 hr after the FM, and one each at 0600 hr, 1000 hr, and 1500 hr during the NM week.

DISCUSSION

This study examined the rhythmic behavior of several species of tropical fiddler crabs under natural field conditions. Early behavioral studies concerning rhythmic activity in *Uca* concentrated on the locomotory activity of crabs in laboratory containers (see Palmer, 1973, for review). Recent studies have revealed a far greater variability within a population than was previously indicated. Some individuals exhibited tidal rhythms, others diurnal rhythms and still others no apparent rhythm at all (Zucker, 1973; Lehmann, Neumann, Kaiser, 1974; Rawson and DeCoursey, 1976). Since so much variability does exist, the degree of synchrony within a population is of interest. Furthermore, studies of rhythmic activity performed under natural field conditions might shed some light on the adaptive significance of rhythmic cycling for the crabs.

Previous studies suggested that courtship activity by tropical males cycled on a semi-monthly basis (Crane, 1958; Feest, 1969; von Hagen, 1970; Zucker, 1976). Two of these studies (Crane, 1958; and von Hagen, 1970) used a criterion of 50% or more of the males waving to indicate courtship peaks. Using this criterion and pooling all courtship activities (waving and waving-and-feeding) only *U. m. terpsichores* in this study shows semi-monthly peaks of courtship. Previous work with *U. m. terpsichores,* which utilized a different method of observation, showed courtship activity occurring about equally after both the FM and NM (Zucker, 1973, 1976). However, the present study revealed that significantly more males were courting around the FM than the NM in all three species. Also, the degree of synchrony during peak courtship periods was far better than 50%. In all three species 78% or more of the adult males were engaged in courtship activities (waving and waving-and-feeding) during the same low tide period, one to three days following the FM. Nevertheless, in the present study, *U. m. terpsichores* males showed considerable courtship after the NM. This species, too, was the only one of the three in which as many males emerged several days after the NM as the FM. Thus, while *U. latimanus* and *U. beebei* males exhibited a distinct monthly cycle of courtship activity, *U. m. terpsichores* tended toward a semi-monthly cycle.

The relatively large amount of courtship activity performed by specimens of *U. m. terpsichores* following the NM may be explained by their location in the upper-most part of the intertidal zone. As shown in Figure 1 and mentioned in the results, the crabs did not emerge when the previous high tide did not cover them, which occurred around the neap tides. Apparently, during those days the dry substrate forced these semi-aquatic animals into estivation. Consequently, *U. m. terpsichores* was active only 7 days (the 2 rainy days also inhibited activity) during the 2 weeks around the FM, while the lowest of the three species, *U. beebei,* was active a full thirteen days during the same period (Fig. 2). Courtship activity during the NM period by male *U. m. terpsichores* may have compensated for the short activity period available to them during the FM weeks. Also associated with the relatively few active days available to *U. m. terpsichores* during the FM period is the greatly reduced amount of feeding at this time compared to the other two species. Peak feeding periods are early morning hours on days preceding both the FM and NM for *U. latimanus* and *U. beebei* but only before the NM for *U. m. terpsichores.* Over the entire month of observation, only 15% of the *U. m. terpsichores* males engaged in pure feeding activity, while more than twice that percentage did so in the other two species (Table II).

The reduced feeding activity by *U. m. terpsichores* may be due to any of several factors. *U. m. terpsichores* is the smallest of the three species and may, therefore, require less food for maintenance. More likely it "catches-up" on feeding by waving-and-feeding more frequently than the other two species (Table II). It may also engage in more feeding at times other than when observations were made. Agonistic activities are also usually confined to the early hours of the low-tide periods, especially on those days in which courtship will later predominate. Thus, the small amount of agonistic activities (as revealed in Table II) is somewhat misleading. When the males first emerge after the tide recedes they are cryptically colored and usually engage in some feeding. If courtship will take place later in the period, much aggression occurs presumably to secure an area for display free of other crabs, both males and females (Zucker, 1977). Once courtship starts (when these data were obtained) the females have been displaced from the males' display grounds and the males are well spaced; little aggression occurs.

One additional behavior, hood-building, was highly synchronized within the male populations. The greatest hood-building activity took place during several days just after the FM, coinciding with peak courtship activity in all three species (Figs. 2 and 3, Table III). Of the three species, only *U. latimanus* exhibited a near perfect correlation between pure courtship (waving only) and hood-building (Table III). In fact, virtually all courting *U. latimanus* males constructed hoods earlier in the low-tide period. *U. beebei* showed the smallest ratio of hoods to courting males, with *U. m. terpsichores* intermediate (Table III). In other words, in all three species only courting males build hoods, but only in *U. latimanus* is hood possession an integral part of courtship. A fuller account of the function of hoods is given elsewhere (Zucker, 1974; and in preparation). Hence, feeding, courtship, and hood-building activities were synchronized among males in all three species. *U. latimanus* males showed the greatest degree of synchrony and *U. beebei* the least.

Barnwell (1968) suggested that differences in the timing of courtship between species might act as a species isolation mechanism. He based this idea on Crane's (1958) observation that primitive species courted earlier in the morning than advanced species. Since all three species in the present study courted at the same time and were found intermingling on the same flat, limited courtship periods probably do not serve as an isolation mechanism here. The number of receptive females in a population and their reproductive behavior are probably two important factors. The ratios of males to females observed in this study for *U. m. terpsichores, U. latimanus* and *U. beebei* were 1.1:1, 2.6:1, and 0.6:1, respectively. From these observations there is no way of knowing how many females were sexually mature and sexually receptive. It is probably valid to assume that for the three species the proportion of receptive to nonreceptive females was similar. Thus, there were probably many fewer receptive *U. latimanus* females per courting male than in *U. beebei,* with *U. m. terpsichores* falling in between. Crane (1975) has stated that wandering females are sexually receptive. In this study, five of the eight observations of wandering females occurred on days just after the FM. If these females were sexually receptive, then they were synchronizing their reproductive activities with that of the males. However, there have not been any studies correlating wandering activity and ovarian maturity. Several studies have examined ovarian and egg development in relation to lunar phase (von Hagen, 1962, 1970; Feest, 1969; Zucker, 1973). Two of the studies concerning ovarian development (von Hagen, 1962, in *U. tangeri;* Zucker, 1973, in *U. m. terpsichores*) revealed that the ovaries were most highly developed around the FM. However, much individual variation existed. In *U. m. terpsichores* some females exhibited well-developed ovaries during each lunar phase (Zucker, 1973). In *U. m. terpsichores,* at least, males which court at times other than the FM period *(i.e.,* around NM) still have the opportunity of attracting and mating with a sexually receptive female. Von Hagen (1970) showed that females of the seven Trinidad species of *Uca* reach two ovarian maxima: the greatest on days just before the NM, the second on days just before the FM. No indication of differences among the species were given. Males of four of the species showed courtship occurring around both FM and NM. Three of the species showed no distinct rhythm of courtship. Since *U. latimanus* males in the present study almost never engaged in courtship after the NM, it would be interesting to see whether females of this species show only a single ovarian maximum around the FM period with considerably less population variation than the previous studies revealed. These few studies indicate, not surprisingly, that the two sexes are apparently synchronizing their reproductive activities.

The ratio of males to females in a population may influence the degree of synchronization of male courtship. The apparently low ratio of receptive *U. latimanus* females to courting males may be a factor in selecting for the high degree of male synchronous courtship activity revealed in this species. There is some evidence that male courtship not only stimulates females to mate but also stimulates neighboring males to wave in concert and to court more vigorously (Gordon, 1958; Salmon, 1965). The presence of virtually all adult males vigorously courting at one time might insure that the greatest possible number of the relatively few females are stimulated to mate each month. Conversely, the males belonging to the species with the greatest proportion of females (*U. beebei*) showed the least concentration of courtship activities to specific days of the month (Fig. 2). In fact, compared to the other two species, only half as many *U. beebei* males (17%) devoted their time to pure courtship (waving only) (Table II). Apparently, *U. beebei* females could be stimulated to mate with males which invested less time in waving and more in feeding activities than the other two species (Table II). The observations of mated pairs of *U. beebei* on the surface at various times throughout the month also suggests that female specimens of *U. beebei* may show more variability in their time of receptivity and, perhaps, require less premating stimulation *via* courtship. Normally, males must induce females *via* vigorous courtship to follow them into their burrow, where mating typically occurs.

Courtship activities in males and females are highly synchronized and timed to coincide with the spring tides occurring several days following the FM. At this point it is only possible to speculate on the reasons for reproductive activity occurring around the FM rather than during some other lunar phase or environmental event. The availability of food for the resultant larvae has been suggested as the key to the timing of reproduction in annually reproducing marine crabs (Boolootian, Giese, Farmanfarmaian, Tucker, 1959). Seasonal reproduction is apparently not involved in tropical *Uca* since crabs of all sizes (and ages) were observed on the flats throughout the entire year during previous studies (1970–1971, personal observation). Thus, an adequate supply of food must be available throughout the year. However, it is entirely possible that production of the planktonic food sources for larval *Uca* also show cyclic peaks which coincide with the time of *Uca* larval release each month.

A second hypothesis is that mating on days just after the FM insures larval release during the most favorable tides. The most favorable tides should be the highest or spring tides since neap high tides did not always cover the flat. Releasing larvae during the neap tides would require ovigerous females to make a dangerous (?) trek down to the water's edge. According to Feest (1969), larvae of tropical *U. tangeri* and *U. annulipes* are released about two weeks after egg-laying. If the eggs are laid shortly after mating (as Christy, 1978, observed in *U. pugilator*), then they would be ready to be released into the ocean two weeks later, during the following spring-tide period. Likewise, the timing of courtship and the subsequent larval release might provide the larvae with optimal tidal currents several weeks later to transport them back to suitable adult habitats.

Christy (1978) has recently proposed this hypothesis to explain semi-monthly releases of *U. pugilator* larvae off the west coast of Florida. Courtship and mating also peaked twice monthly during the quarter moons (neap tides). Though individual females apparently released larvae only once a month (about two weeks after mating), some did so during each of the quarter moon neap-tide periods. From laboratory studies, he estimated that young crabs would be ready to settle on an intertidal flat about three weeks later, *i.e.*, during spring tides. Spring-tide currents would help ensure up-estuary transport toward suitable adult habitats (Christy, 1978). The same explanation may be true for the three tropical species reported here even though courtship activities are displaced a week from the neap-tide period to the spring-tide period. The warmer waters in Panama as compared to Florida might speed up larval development to the point where young crabs are ready to settle two, rather than three, weeks after release, thus during the next spring tides.

Another possibility is that the timing is not influenced by some requirement of reproduction but rather a requirement of feeding by the adults. The spring tides (when courtship occurs) always coincide with low tides occurring in the late morning, just prior to the noon hour. At this part of the day the substrate is fairly dried out due to the high temperatures, humidity decrease and extreme low tide which thoroughly drains the flat. The dried mud-flat may not be appropriate for efficient feeding (filtering organic matter left on the mud by the ebbing tide). If the crabs are restricted to feeding on the wetter substrate, then courtship would be allocated to mid-day hours, which occur on days just after the NM or FM. Some evidence for this is seen from a previous experiment with *U. m. terpsichores* housed in an outdoor "crabbery" in Panama (Zucker, 1976). The tidal cycles could be manipulated but all other environment factors remained normal (L/D cycles, weather, lunar influence). Crabs maintained on a precise 24 hr tidal cycle with low tide always falling at mid-day and midnight took four weeks to entrain to this tidal cycle, but once they had done so, courtship occurred daily from then on. Crabs maintained on an early morning low-tide period never courted, but fed instead (Zucker, 1976).

All these hypotheses provide explanations for why courtship (and reproduction) should occur around the spring tides. However, there are two spring-tide periods each month, following the NM and the FM. Yet, all three hood-building, Panamanian *Uca* species emphasize the FM spring tides for courtship activities. One spring tide each month is usually more extreme than the other. Every seven months the more extreme spring tide of the month switches (from Tide Tables—USCGS). Over a period of years any given month will have extreme tides after both NM and FM. Thus, there appears to be no particular advantage in timing reproductive activities to either the FM or the NM. The answer to the question of why these *Uca* males court around the FM period must await further studies concentrating on reproduction, both at the level of the crab's behavior in the field and its physiology.

I wish to thank Drs. M. Salmon and P. DeCoursey for reading and criticizing an earlier version of this paper. This study was carried out at the facilities of the Smithsonian Tropical Research Institute's Naos Island marine laboratory in Balboa, Canal Zone. This work was supported by National Science Foundation grant BMS 75-01124.

SUMMARY

1. The degree of synchrony with which three tropical fiddler crabs, *Uca musica terpsichores, U. latimanus* and *U. beebei*, perform courtship, feeding and hood-building activities was studied.

2. Courtship activities cycled on a monthly basis. Significantly more males of all three species courted during the week of the full moon when compared to the

week of the new moon. Virtually no courtship occurred around the quarter moon periods. During peak courtship periods, 78% or more of the males of each species were displaying at the same time.

3. Hood-building also peaked around the full moon and was performed only by courting males. Only a small percentage of courting *U. beebei* males built hoods, while virtually every courting *U. latimanus* male possessed one. An intermediate number of *U. musica terpsichores* built hoods.

4. Almost all of the observed females were feeding. Wandering females (presumably sexually receptive) were observed almost exclusively during the same low tide hours in which males were courting.

5. Both males and females, therefore, appear to be synchronizing and concentrating their reproductive activities to the full-moon period.

Note added in proof

Observations made at LaBoca, Balboa, CZ during July and August 1978 revealed peak courtship occurring during the spring tides following the NM and not following the FM as had been the case during the summer of 1975. This was especially evident in *Uca latimanus*. A check of the 1975 tide tables (USCGS) revealed that the spring tides following the NM were more extreme than those following the FM during the observation period. That is, courtship occurred during the less extreme of the spring tides. In 1978, the reverse occurred, with the extreme tides following the FM. Courtship in 1978, therefore, still took place during the less extreme spring tides, but this time after the NM. This observation lends support to the hypothesis that courtship is timed to permit optimal larval release or larval recruitment back to the flat. Eggs fertilized during the less extreme spring tide period could be ready for release two weeks later during the more extreme tides and/or ready for settling back on the flat four weeks (one lunar cycle) after that. Thus, courtship in these tropical *Uca* appears not to be tied to a particular lunar phase but rather to the less extreme spring tide period each lunar month.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

Abstracts are arranged alphabetically by first author. Author and subject references will be found in the regular volume index, appearing in the December issue.

GENERAL SCIENTIFIC MEETINGS

AUGUST 21–23, 1978

Studies on sex steroids in the gonads of marine organisms and the frog (Rana pipiens). CHRISTOPHER A. ADEJUWON, S. J. SEGAL, AND S. S. KOIDE.

Although the presence of sex steroids (testosterone, estradiol-17β, and progesterone) together with their various metabolites has been qualitatively demonstrated in the gonads of various aquatic animals, information on their quantitative levels is lacking. Previously, these steroids have been demonstrated with histochemical and isotope dilution techniques which do not yield quantitative information. In the present study, highly specific radioimmunoassay methods have been used to determine testosterone and progesterone levels in the gonads of the frog (*Rana pipiens*) and several aquatic animals.

The testosterone content in the testis of the frog (*Rana pipiens*) was 24.46 ± 2.57 ng per g, in the testis of the dogfish (*Squalus acanthias*) 4.2 ± 0.003 ng per g, and in the polychaeta (*Chaetopterus pergamentaceus*) 0.24 ± 0.05 ng per g. Testosterone was not detectable in the testes of the following animals: starfish (*Asterias forbesi*), surf clam (*Spisula solidissima*), sea urchin (*Arbacia punctulata*), and in the ovatestis of grassworm (*Leptosynapta inherens*). The ovatestis of *Leptosynapta inherens* contained 0.39 ± 0.09 ng of progesterone per g of wet tissue while the mature ovary of the frog (*Rana pipiens*) contained no detectable progesterone.

The present results suggest that the testes of vertebrates may contain more testosterone than those of invertebrates. The high content of testosterone and non-detectable amounts of progesterone in the testis of *Rana* suggest that there may exist an alternate pathway in the biosynthesis of testosterone.

Supported in part by a grant from The General Service Foundation.

Time lapse microcinematographic studies of cell motility in the embryonic yolk sac of Fundulus heteroclitus. PETER B. ARMSTRONG.

Active motility of tissue cells represents an important process in the embryogenesis of animals. The present study is a time lapse microcinematographic analysis of the active motility of the cells of the yolk sac of the living *Fundulus* embryo. In the teleost, the yolk sac lies peripheral to the body of the embryo proper and consists of a space roofed by an epithelium, the enveloping layer, and with the yolk syncytial layer as the floor. The yolk of the *Fundulus* embryo is so transparent that cell migration in the yolk sac can be studied by direct microscopic observation of the living embryo with phase contrast optics. The motile cells of the yolk sac space include the yolk sac amebocyte, a cell seen first during epiboly (stage 15), and the neural crest, which begins migrating from the embryo at stage 19. The two cells show marked differences in motile behavior. The amebocytes are compact, rounded cells that migrate rapidly by the extension of short lamellipods. The amebocytes migrate in an apparently undirected fashion and invade the embryo proper when they encounter it, moving between cells of the lateral mesoderm. The neural crest cells migrate by the slow extension of elongated polymorphic processes, followed by the rapid movement of the cell toward the extended process. Movement frequently is perpendicular to the predominant long axis of the cell: processes are extended obliquely forward, alternatively from opposite ends of the cell, so the path described is a zig-zag to either side of the overall direction of movement. Although neural crest cells show considerable irregularity in their movement, the predominant direction is away from the embryo proper.

Supported by funds of the University of California Cancer Research Coordinating Committee and NSF Grant #PCM77-18950.

Preliminary studies on the closure of the optic vesicle of Loligo pealei *embryos.* John M. Arnold and Lois D. Williams-Arnold.

The optic vesicles of squid arise from two oblong placodes on either side of the embryo. At the edge of each placode a tissue fold first grows outward, then toward the placode's center. The future retina forms from the inner wall of the vesicle. Using the scanning electron microscope, the cells at the leading edge of the oval constriction are seen to be circumferentially spindle-shaped with surface folds paralleling their long axis, thus appearing under tension. During optic vesicle closure, thin, sheet-like cytoplasmic projections connect the leading edge to the future retina below. These projections seem to be under tension because they have radially-oriented, longitudinal surface folds, precede the leading edge, and slant outward at sharp angles. Although variable in size and number, these projections are always present. These observations suggest that optic vesicle closure is caused by first, a purse-string effect of the leading edge; secondly, pulling of the cytoplasmic projections between the future retina and leading edge; or thirdly, a combination of both mechanisms.

Microsurgical experiments were performed when the outer optic vesicle wall was approximately half closed and earlier when the fold at the edge of the placode was growing outward. A radial cut in the former case immediately opened into a triangular wound. A cut made outside and parallel to the leading edge caused the leading edge to immediately contract inward and assume a straight line the same length as the cut. Early folds similarly cut before closure had begun did not gape open, nor did the leading edge suddenly advance. Cytochalasin B caused partially closed optic vesicles to rapidly evert.

It is concluded that the optic vesicle is closed by circumferential tension at its leading edge with the cytoplasmic connections between the future retina and the leading edge possibly acting as guides.

Supported by N. S. F. Grant #PCM 76-19301.

A salt marsh predator-prey relationship: attack behavior of Carcinus maenas (L.) and defenses of Ilyanassa obsoleta *(Say).* Linda R. Ashikenas and Jelle Atema.

Salt marsh mudsnails (*Ilyanassa obsoleta*) burrow rapidly into the sediment when they chemically sense body juices from a nearby wounded conspecific. This behavior would be adaptive if the attack and feeding techniques of the predator result in the release of an alarm stimulus. We investigated predation success rates and feeding methods of *Carcinus maenas* (the green crab) on different sizes of *I. obsoleta*.

Six crabs were maintained in individual 100 l aquaria and were presented with pairs of similarly sized mudsails. Observations were made of techniques of shell attack, shell breaking, and feeding. The responses of the unmolested snail were used as an additional indication of attack success. Snail size classes of 10 to 15, 15 to 20, and >20 mm shell length were tested. Crabs used two methods of attack: (1) puncturing and subsequently shearing the spire; (2) chipping away the outer lip of the aperture. For all snail sizes, 77% of the successful attacks were to the spire. The crabs fed after initially breaking the shell (which liberated body juice), then returned to further cracking the shell. Unmolested snails burrowed in response to alarm juices and were attacked only twice. Crabs opened the smallest mudsnails easily: 17/24 (71%) of this group were consumed. Success rates were significantly lower in the two larger size classes, 2/23 (9%) and 7/25 (28%) respectively (chi-square, $p < .001$). However, among individual crabs, success rates varied greatly with snails larger than 15 mm.

These preliminary findings suggest that the attack and feeding techniques of *Carcinus maenas* release an alarm stimulus, thereby eliciting burrowing by *Ilyanassa obsoleta*. Thus the alarm response can be an adaptive predator defense. Large mudsnails possibly have a size refuge from green crab predation. We are presently investigating predation time budgets for *C. maenas* and other potential predators and their correlation with response times for the alarm behavior.

Research partially supported by EPA #R803833 and DOE #EY-76-s-02-2546 to J. Atema and by the Boston University Marine Program.

Microtubule-inhibiting drugs alter chemoreceptor function in olfactory sensilla of the lobster. JELLE ATEMA, DEMETRIA JAMES, AND SUSAN OLESZKO-SZUTS.

It has been postulated that microtubules are actively involved in sensory information transmission, acting as a fast intracellular relay system (Atema 1973, *J. Theor. Biol.*, **38**: 181; 1975, in Borgers and deBrabander, *Microtubules and Microtubule Inhibitors:* 247). The distal segment of ciliary sensory cells provides a system where this theory can be tested: altering or destroying the microtubules with drugs should change or block the sensory process. Thus far, only mechanoreceptor cells have been tested. Moran and Varela (1971, *Proc. Natl. Acad. Sci.*, USA, **68**: 757) found that colchicine and vinblastine disassembled microtubule structure and simultaneously reduced the spiking activity of the receptors.

In mechanoreceptor cells, however, microtubules may act as stiff rods which carry out the mechanotransduction process. A better system would be receptors which do not interpret mechanical signals. We used the olfactory (antennular) receptor cells of the lobster, *Homarus americanus,* where 800-μ long, finely-branched distal segments of about 400 bipolar receptor cells are packed inside a spongy cuticle. The proximal segments, cell bodies and axons are located inside a dense cuticle. These anatomical features allow easy drug access to distal segments and simple neurophysiological recording from corresponding axons a few centimeters away.

In control experiments, 10 antennules of intact lobsters were bathed in artificial sea water for 2 to 3 hrs. The antennules were cut off and their neurophysiological response to standard mussel extract recorded. Eighty-four percent of the spontaneously active receptors showed significant responses to the test stimulus. In identical procedures, 10 antennules were bathed in 10^{-3} M colchicine for 3 hr in darkness and 10 in 10^{-4} M podophyllotoxin for 1.5 hr; receptor responses were measured as in the controls. In these experiments, only 25 and 19%, respectively, of the spontaneously active cells responded to the stimulus. The number of spontaneously active receptors was the same for all three treatments.

These results suggest that the drugs did not affect the spontaneous activity of the receptors, but did interfere with distal segment processes. Although not proving an information transmission function for microtubules, these experimental results encourage further work in that direction.

Vital staining of invertebrate mucous secretions by Janus green-B. BETSY G. BANG AND FREDERICK B. BANG.

In earlier studies we had found that the intracellular mucus of the free urn cell of *Sipunculus nudus* stained vitally with methylene blue, while the emergent mucus failed to stain. In using Janus green-B to identify the mitochondria of the cell we found by chance that this dye stained the mucus during secretion, both with the violet reduced form and the bluegreen oxidized form of the dye, and that the dye itself was a mild mucus stimulus. One of the strongest stimuli to mucus secretion *in vitro* was heated human serum. This summer we have found that preincubation of urn cells with glutathione very greatly enhanced the response to heated serum, but that if the cells were incubated with Janus green before incubation with glutathione, the response to heated serum was completely inhibited.

Responses of representative species of the phyla Cnidaria, Ctenophora, Echinodermata, Mollusca, Annelida, Platyhelminthes, Hemichordata, and Chordata to Janus green were studied by immersing each test animal in 1:100,000 seawater dilutions of the dye, then after 20 min returning them to seawater. All 12 species within these phyla responded by producing mucus; this varied in time of response, amount, quality, and in staining with reduced, oxidized, or leuco forms of the dye. Most studied was the ctenophore *Pleurobrachia,* in which mucus secretion seems not to have been previously noted. Dye was immediately concentrated in interctene glands, which then produced very sticky blue mucus which inhibited ctene motility. As in urn cells, heated human serum induced production of clouds of mucus. Unlike *Pleurobrachia*, the mucus induced by the dye in *Cratena* and other small nudibranchs was not sticky and did not inhibit mucociliary transport.

Janus green-B has not been previously recognized as either a vital stain of mucus or a mucogogue. It may prove useful in identifying otherwise invisible mucus and in classifying mucous responses in other invertebrate species.

The effects of fatty acids on the binding of saxitoxin to nerve membranes. JESSE BAUMGOLD, SUSUMU TERAKAWA, AND SUSAN SWARTZ.

The binding of ^{3}H-saxitoxin to lobster nerve membranes to which fatty acids had been added, was studied. The binding studies were performed using the Millipore filtration method. The fatty acids were added by homogenizing the membrane suspension in a homogenizer containing dried fatty acid. We found that the saxitoxin binding was sensitive to the degree of unsaturation of the added fatty acid. When added at a protein to fatty acid ratio of 4 to 1, arachidonic acid (20:4) inhibited the binding completely, followed, in decreasing order of effectiveness, by linolenic acid (18:3), linoleic acid (18:2), and oleic acid (18:1). The fully saturated fatty acids tested had no effect on the binding, and the methyl ester derivatives of the fatty acids had very little effect on the binding.

The electrophysiological aspects of these results were studied using squid giant axons that had been exposed to aqueous suspensions of arachidonic acid. These axons were found to lose excitability irreversibly. After the arachidonic acid treatment, the threshold for excitation rose gradually, and the resting potential depolarized slightly. In a voltage clamped axon, the arachidonic acid pretreatment reduced and eventually reversed the initial inward current. The effects on the delayed outward current were relatively small. Finally, arachidonic acid pretreatment was found to shift the K_d of the dose-response curve for saxitoxin to the right.

These data suggest that the saxitoxin binding protein contains a hydrophobic region capable of interacting with fatty acids. When these fatty acids contain double bonds, a conformational change is evoked in the protein which is manifested as a reduction in the saxitoxin binding capacity of that protein.

Labyrinthula *cells and matrix: an interacting motile system.* EUGENE BELL, STEPHANIE SHER, DENNIS SOLOMON, AND NORIO NAKATSUJI.

Labyrinthula is a colonial marine protist consisting of cells and a surrounding matrix ("slimeways") which the cells secrete. Cell translocation is dependent on matrix, since isolated cells are stationary until they generate matrix. In time-lapse films we observe the extrusion and fusion of matrix filaments which move actively on a substrate and, for the first time, lamellipodial behavior exhibited by the matrix particularly at growing points. Cells have no visible means of locomotion, that is, no podia, cilia, or flagella, yet glide in either direction in a slimeway.

Colonies stain strongly with fluorescein conjugated antiactin and antimyosin antibodies. The antimyosin antibody staining is heaviest along the edges of slimeways giving the appearance, sometimes, of a pair of parallel rails. In the region of a cell, the "rails" are seen applied only to that portion of the cell perimeter in contact with the "slimeway." In the zones of contact between cells in a "slimeway" staining, as in the cells themselves, is dim. The "rails" are not seen in controls prepared with normal rabbit serum or with competing antigen. In cultures stained with antiactin antibody both cells and slimeways stain relatively uniformly. Slimeway edge definition is observed however with polarization optics. Although we have not separated matrix from cells, a protein present in whole colony extracts co-migrates electrophoretically with chicken gizzard myosin.

We hypothesize that cell translocation may depend on an actomyosin system in which one component, myosin, resides in the slimeway, and the other, actin, on the cell surface. An alternative hypothesis, that cell translocation is due to the matrix alone seems less tenable since 6 nm filaments are oriented parallel to the long axis only and no "contractile ring" structures have been seen in the matrix at the training end of cells.

Work supported by a grant from M.I.T. Sea Grant Program.

Electrotonic junctions between Fundulus *blastomeres: reversible block by low intracellular pH.* M. V. L. BENNETT, J. E. BROWN, A. L. HARRIS, AND D. C. SPRAY.

Single blastomeres were isolated from cleavage stages of the killifish *Fundulus heteroclitus* and reaggregated in pairs in double strength Holtfreter's saline. Each cell of a pair was penetrated by a current and a voltage electrode, and non-junctional and junctional resistances were calculated from input and transfer resistances. The pairs become electrotonically coupled in 5 to 20 min and gap junctions are formed between them. Bathing in saline equilibrated with 100% CO_2 (pH 5.8) decreases junctional conductance 100-fold or more within 20 sec. Nonjunctional conductances are decreased to much lesser extent. On return to normal saline junctional conductance rapidly returns (100-fold change within 40 sec). Similar but slower effects are produced by saline in which Na acetate or propionate replaces NaCl. Acetate and propionate salines may decrease junctional conductance when pH is 7.4 to 7.8, but in some preparations pH has to be reduced to 6 or 6.6. Bathing in pH 6 salines with impermeant buffers HEPES or phosphate for 10 to 15 minutes has no effect on junctional conductance. These data indicate that acidification of the cytoplasm caused by permeation of CO_2, acetic acid, or propionic acid decreases junctional conductance. To determine if these agents act indirectly by increasing intracellular free Ca, one of a pair of coupled cells was injected with aequorin and light emission was measured during brief applications of CO_2 or acetate salines. No change in background emission occurs as junctional conductance falls. In about half the experiments, there is a delayed increase in light emission which may occur while junctional conductance is increasing back towards the initial value following return to normal saline. We conclude that low intracellular pH decreases junctional conductance without increasing intracellular free Ca.

Optical recording of neuron activity in Hermissenda, Hirudo, *and* Navanax *ganglia.* M. B. Boyle, S. Lesher, and L. B. Cohen.

Optical methods for monitoring neuron activity which were first used with supraesophageal ganglia from *Balanus nubilus* (L. B. Cohen, A. Grinwald, and S. Lesher, *Neuroscience Abstracts,* 1978) were tested on ganglia from three additional species, one an annelid and two molluscs. Using the absorption change of the dye NK2367, a 32X (0.6 NA) objective, and a 10 × 10 array of photodiodes positioned in the objective image plane, optical signals that correspond to action potentials were detected without difficulty in buccal ganglia of *Navanax inermis,* and in pleural and pedal ganglia of *Hermissenda crassicornis.* Although the presence of the unstained ganglia from these species reduced the light reaching the photodetectors by 70 to 90%, their high opacity (relative to *Balanus* and *Hirudo* ganglia) did not seem to cause problems in detection. Similar optical signals were seen in some experiments on segmental ganglia from *Hirudo medicinalis,* although in others the signals were small. While we plan further experiments to try to improve the reliability of the technique using *Hirudo* ganglia, the overall results suggest that optical methods may be rather generally applicable in invertebrate central nervous systems.

Supported by N.I.H. Grant #NS 08437.

Chemical communication in catfish: Stress-induced changes in body odor. Bruce Bryant, Randall Elgin, and Jelle Atema.

Changes in metabolic processes often result in changes in body odor, which in turn could be used for conspecific and interspecific communication. This possibility has been suggested for catfish. The aim of the present project has been to show that changes in the chemical composition of skin mucus occur during certain behavioral interactions in the catfish, *Ictalurus nebulosus.*

It was first established that catfish can chemically sense a change in body odor of another catfish which has experienced the stress of a territorial fight. In subsequent experiments, individual catfish were introduced into aquaria, where an established resident usually fought them until defeat. Samples of mucus from each fish were taken before and after the interaction. These samples were chromatographed and amino acids visualized with fluorescamine. Initial mucus compositions showed individual patterns which were relatively constant over time. After fighting, the mucus composition changed and then returned to normal within 16 to 24 hr. These changes were reproducible with further stress. Neither residency nor fight outcome seem to influence these changes in any obvious or consistent way; at present, it appears that the observed change is stress related. Lactate, an expected exercise metabolite, was not detected.

In conjunction with behavioral observations, these results provide chemical evidence that catfish sense stress in a conspecific. Whether or not this capability is used under natural circumstances remains to be shown.

Cell surface properties of larval and adult cell types in the marine sponge Microciona prolifera. WERNER BURKART, TRACY L. SIMPSON, AND MAX M. BURGER.

Surface properties of *Microciona prolifera* cells were characterized (a) by studying aggregation of larval cells and (b) by determining adult cell distribution in a dextran/polyethylene glycol partition system.

Larvae are released during a short period in summer. Sponges were kept in a small, aerated volume of seawater overnight. The free swimming larvae were harvested by filtration with a 25 μm mesh cloth and then dissociated in calcium magnesium free seawater by triturating with a 100 μm fire polished pipet. After shaking for 5 hr at 4° C, the core cells were spun down. A second centrifugation of the supernatant at higher speed yielded a pellet containing the flagellated cells. In the aggregation assay, the larval cells formed loose aggregates which were easily dissociated. Addition of the larval supernatant promoted formation of very stable aggregates. When *Microciona* aggregation factor (MAF) was added, almost all larval cells became enclosed in its matrix. MAF was preincubated at room temperature with the flagellated larval cells to test for possible degradative or inhibitory action of the larval surface on MAF. Preliminary experiments showed no decrease of MAF activity in subsequent aggregation assays with adult cells.

The surface properties of adult *Microciona prolifera* cell fractions isolated by a scaled-up Ficoll gradient technique were characterized using cell partitioning in two-polymer aqueous phase systems containing 4.5% dextran T500 and 3.8% polyethylene glycol. The partition coefficient of sponge cells between these two phases can be shifted several orders of magnitude by raising the phosphate concentration. Mechanically dissociated cells and ^{125}I labeled MAF have a similar distribution pattern whereas chemically dissociated cells have a lower affinity to the polyethylene glycol phase at intermediate phosphate concentrations. Additional partition experiments with small MAF-coated dextran beads suggest that MAF is a major factor in determining partition coefficients of sponge cells. Different cell types from chemically dissociated cell suspensions showed slight differences in partition coefficients leading to the possibility of cell separation by counter-current distribution.

Supported by the Swiss National Science Foundation.

Respiration rates of oiled sediment cores from Buzzards Bay, Massachusetts. COLLEEN M. CAVANAUGH AND MARILYN J. JORDAN.

The effect of No. 2 fuel oil on benthic respiration and bacterial numbers was measured in mud sediment cores collected by divers in the Northwest Gutter of Naushon Island near Woods Hole, Massachusetts. Three sets of 12 intact sediment cores with the overlying water were collected in August, September and October, 1977, and maintained in the laboratory for four to six weeks under running seawater at ambient seawater temperatures; approximately 22° C for set I, 20° C for set II, and 17° C for set III. Six of the sediment cores from each set were exposed to a 0.2% mixture of No. 2 fuel oil in seawater for 4 hr. Concentrations of oil in the top 1 cm of sediment one day after exposure ranged from 12 to 480 μg/cm^3 (1 cm^3 = 0.3 g dry weight). The concentrations of oil declined by 60 to 96% within 6 weeks.

Oxygen concentration was monitored using an oxygen electrode on 3 oiled and 3 control cores for each set. Measurements were made the day of collection before oiling, 1 day, 1 week, 3 to 4 weeks, and 6 weeks after oiling. Sediment respiration was corrected for plankton respiration in the overlying water by measuring oxygen consumption of a control core containing just seawater and subtracting the volume corrected rate from the rates measured for the sample cores. The rate of oxygen consumption was calculated by linear regression of oxygen concentration vs time. Direct counts of bacteria were made using the acridine orange epifluorescent technique.

The respiration rates and bacterial numbers of the oiled sediments were not significantly different than those of control sediments at $p = 0.05$. Sediment respiration rates of each set generally increased in the first one to three weeks following collection, then declined. Benthic respiration rates (reported as mg O_2 m^{-2} hr^{-1}) over the 6 weeks of measurements ranged from 68.5 to 99.4 for set I, 34.5 to 71.7 for set II, and 33.1 to 82.7 for set III. Numbers of bacteria ranged from 5.8 to 8.9×10^9 cells/cm^3. Similar respiration rates and bacterial numbers were measured in field samples of mud sediment cores taken in spring and summer, 1977, from the same area.

This research was funded by NOAA under contract 03-7-022-35133, and by DOE under contract E(11-1)-2989 to the Marine Biological Laboratory.

Neurotransmission between rods and horizontal cells in the skate retina. Richard L. Chappell, John E. Dowling, Harris Ripps, and Samuel M. Wu.

Neurotransmission between photoreceptors and horizontal cells in the vertebrate retina involves the discharge of a depolarizing agent in darkness and its suppression by light; the light-induced reduction in transmitter release causes the horizontal cell to hyperpolarize. Both L-aspartate and L-glutamate exert a depolarizing effect on horizontal cells in a variety of species while carbachol, an acetylcholine agonist, has a similar effect in carp. We tested the relative effectiveness of these three agents on horizontal cell responses recorded intracellularly in the isolated all-rod retina of the skate. Drugs were applied in Ringer's solution from atomizers that had been calibrated using radioactive tracers. L-aspartate (0.9 mM) depolarized horizontal cells to the same level above the initial resting potential regardless of whether it was applied in the dark, while the cell was hyperpolarized by an adapting light, or during application of Co^{++} (2.5 mM), which blocked receptor transmitter release. Aspartate also substantially reduced light-induced horizontal cell responses. L-glutamate in higher concentrations (1.6 mM) had a smaller depolarizing effect on horizontal cells in both dark- and light-adapted retinas; it only slightly reduced the responses to test flashes, and its effect on the cobalt treated preparation was minimal. Recovery from L-glutamate depolarization was slower than that after L-aspartate, indicating that its uptake is not greater than that for L-aspartate. Carbachol produced a slight hyperpolarization of some horizontal cells in the dark-adapted retina, but had no effect in light-adapted retinas. Dose-response curves for the amino acids obtained during cobalt blockage show L-aspartate sensitivity to be at least 50 times greater than that of L-glutamate; this result is similar to the findings obtained in cone horizontal cells of the carp. Based on these observations, we consider L-asparate a close analog of the natural receptor transmitter in the skate retina and a strong candidate for that role.

Supported by grants EY-00777, EY-00824, and EY-00285.

Marginal bands of Homarus americanus *coelomocytes: disappearance associated with changes in cell morphology.* William D. Cohen, Iris Nemhauser, and Marion F. Cohen.

Marginal bands (MBs) are regarded as major cytoskeletal components of non-mammalian vertebrate erythrocytes and mammalian blood platelets. As we have shown previously, MBs also occur in some marine invertebrate blood cells, specifically *Golfingia gouldi* erythrocytes, *Limulus* amebocytes, and coelomocytes of several crustacean crab species. To these we now add coelomocytes of the lobster *Homarus americanus*, which contain very distinct MBs about 0.2 to 0.3 μm thick, as seen in phase contrast in cells lysed with Triton X-100 under microtubule stabilizing conditions. Blood samples, obtained rapidly by snipping part of a walking leg, were diluted approximately 1:10 (v/v) into the lytic medium (LyM). In the resulting clumps of lysed cells (clots?), most nuclei were associated with MBs. Squashing of clumps displaced some nuclei, with MBs then appearing as free closed loops or figure-8s. Alternately, fresh blood was diluted approximately 1:20 with marine crustacean Ringer's solution and again immediately diluted approximately 1:10 into LyM, revealing individual lysed cells in which a majority of MBs were elliptical. MB morphology corresponded to that of native coelomocytes, which appeared flattened and elliptical when examined rapidly in Ringer's or (more easily) in Ringer's containing 0.1% glutaraldehyde. MB occurrence was general with respect to lobster size and sex: MBs were associated with at least 90% of nuclei in samples from 5 males and 3 females, 12 to 25 cm in length (rostrum tip to telson edge).

When cells were placed on clean glass substrata (slides) they lost ellipticity within minutes, became spiky, and then spread. In spread cells which were lysed by perfusion with LyM, no

MBs were observed. The apparent *in vivo* MB-lability inspired preliminary low temperature and Ca^{++}-lability tests *in vitro* using lysed cells. These tests were negative under conditions employed. We suggest that a specific MB-disassembly mechanism permits morphological transformation to the spread state.

Supported by NIH grant no. HL20902 from the National Heart, Lung, and Blood Institute, and by City University of New York PSC/BHE Grant #12260, to Dr. W. D. Cohen.

The effect of fertilized detritus fed to juvenile fiddler crabs, Uca pugnax. NANCY J. COPLEY.

Fiddler crabs are major consumers in the salt marsh and are important to its dynamics. Growth rates and behavioral changes while on diets of fertilized detritus were examined.

Specimens of *Uca pugnax* in the lab were observed to ingest dead insects and live *Spartina alterniflora* fragmented in a blender as well as fine particles of detritus. They may, therefore, be considered scavengers rather than strict detritivores. When given a choice between agar media of live and dead *S. alterniflora,* all crabs burrowed only in detritus. High variability resulted from tests between both (a) unfertilized and ureafertilized detritus (U), and (b) control and sewage sludge-fertilized detritus (HF). The nitrogen levels in the U and HF treatments were the same.

Growth rates for each of forty crabs per treatment as measured by carapace width were recorded weekly, along with mortality and moltings. Crabs with treatment U grew more than the other two treatments to a highly significant degree. Controls grew significantly more than HF crabs. After 7 weeks a crab population collected from the same site were 4.5 mm larger than the experimental population, suggesting sub-optimal lab conditions. Although growth of HF crabs was almost nonexistent, mortality was less severe than controls, implying a sublethal effect of sewage contaminants. Heavy metals may have caused this lack of growth.

Behavioral changes due to diet were examined by timing escape responses from a shadow. Control crabs were significantly slower than either U of HF animals.

Nitrogen-enriched detritus stimulates growth of crabs. Something in the sludge fertilizer cancels the effect of the nitrogen addition without affecting survival rates, an example of a sublethal effect.

This work was undertaken while enrolled in the Marine Ecology course, MBL.

Depression of Limulus *ventral photoreceptor sensitivity by phosphodiesterase inhibitors.* D. WESLEY CORSON AND ALAN FEIN.

One of two methyl xanthines or a veratryl-quinoline derivative was added to artificial seawater, buffered at pH 7.0 with 10 mM Hepes and suffused onto voltage clamped photoreceptors from the ventral eye of *Limulus polyphemus.* Application of each of these phosphodiesterase inhibitors to a single cell resulted in a rapid and reversible depression of the photoresponse elicited by dim flashes of white light of constant intensity. Each inhibitor reversibly desensitized the photoreceptor by shifting threshold level response log-intensity curves out along the log-intensity axis. The electrical characteristics of the membranes in the dark were examined in the presence of concentrations of each of the drugs which produced approximately 1 log-unit depressions of sensitivity measured at a criterion response level of 2 nA.

The peaks of outward tail currents following 50 mV, 100 msec depolarizing clamp pulses were reduced by $37 \pm 3\%$ ($x \pm$ s.d.), $47 \pm 8\%$, and $84 \pm 14\%$ for Theophylline (5 mM, $n = 3$), IBMX (1 mM, $n = 3$) and Papaverine (50 μM, $n = 2$), respectively. Only the veratryl-quinoline derivative Papaverine appreciably ($66 \pm 7\%$) reduced the late outward current measured during 50 mV, 100 msec clamp pulses while Theophylline and IBMX produced lessor reductions ($12 \pm 2\%$ and $26 \pm 12\%$). In cells where the depression of sensitivity was not completely reversible, recovery of tail currents and late outward currents could occasionally continue to completion. Also, the reverse sometimes occurred. The sensitivity fully recovered while the late outward currents and tail currents only partially recovered. The last two findings suggest that these agents may depress light-induced conductance changes independently of their effects on voltage dependent conductances in the dark.

Ultrastructural study and radioimmunological evidence for progesterone production in the mandibular bland of the lobster (Homarus americanus). ERNEST F. COUCH, C. A. ADEJUWON, S. J. SEGAL, AND S. S. KOIDE.

In 1968 Le Roux (*C.R. Acad. Sci. Paris,* **266**: 1414–1417) described in a large variety of decapod Crustaceans a glandular tissue located near the base of the prominent mandibular adductor muscles. Because of their location, he gave them the name "mandibular organs." It is now clear that in the past this mandibular organ has been incorrectly identified as the Y-organ or the molt hormone producing gland. This error is especially widespread in descriptions made of crayfish and lobsters. It now appears that two pair of glands exist in Crustacea: true Y-organs and mandibular organs (Sochasky *et al.*, 1972, *Can. J. Zool.*, **50**: 993–997). At present essentially nothing is known about the mandibular gland's role in the physiology of Crustacea.

We wish to report here that the lobster mandibular gland has nearly all the ultrastructural features that are normally associated with steroid production. They have, first, a prominent smooth endoplasmic reticulum which often forms a "folded membrane complex"; secondly, mitochondria with tubular cristae; thirdly, lipid droplets; fourthly, a nucleus with a prominent nucleolus and finally, the occasional presence of protein crystals. Furthermore, we have found that, according to radioimmunoassay, the lobster mandibular gland contains progesterone but no testostoerone. The progesterone content of the gland ranged from 0.68 to 2.32 ng per gram of wet weight in the eight specimens analyzed. One specimen had no detectable amount. The blood levels of progesterone in three specimens ranged from 0.040 to 0.058 ng per ml. The progesterone content in immature ovaries ranged from 0.17 to 0.24 ng per gram of wet tissue. The amount of progesterone in the mandibular gland was higher than that found in the blood. At present we do not know the role of the progesterone. It could be an intermediate on a pathway leading to other steroids. The present results are the first demonstration of the presence of progesterone in the mandibular gland of *Homarus americanus.*

Cannibalism in the salt marsh killifish Fundulus heteroclitus. MARGARET DAY.

The effects of adult size and sex and juvenile size and density on cannibalism in *Fundulus heteroclitus* were studied in the laboratory. Adults and juveniles were placed in containers with an opened mussel (*Modiolus demissus*) and detritus as alternate food sources.

The largest adults of both sexes consumed the greatest percentage of juveniles, but the amount of mussel consumed was relatively constant. Females consumed more of both foods than males due to increased energy requirements during the reproductive season. The adults required more time to eat large juveniles (3.0 cm) due to physical limitations. High densities of juveniles resulted in increased cannibalism.

As adult size increases, their food preferences change to include more juveniles, indicating that adults choose to prey on juveniles. Energetically, mussels are the optimal prey. They contain 7% more organic carbon than juveniles; while detritus, which was not ingested, contains 30% less. Prey movement initiates cannibalism. Dead juveniles, often lacking tails, were found. Once the juveniles stopped moving, they apparently were no longer a desirable food source.

It appears that prey movement and the adult's physical ability to consume juveniles determine if cannibalism will occur.

Responses of lobster walking leg chemoreceptors: the role of amino acids, proteins, and live prey body odors as feeding stimuli. CHARLES D. DERBY AND JELLE ATEMA.

Chemicals important in feeding activities of the lobster, *Homarus americanus,* are detected by antennules, as shown in behavioral and electrophysiological studies. The physiological and behavioral functions of walking leg chemoreceptors, however, are not well known.

Extracellular electrophysiological recordings from leg chemoreceptive nerves were obtained in response to 21 amino acids, 1 tripeptide, 3 proteins, 4 sugars, 2 organic acids, 2 live prey body odors, and 1 prey tissue extract. Intensity of response was measured as increase in number of spikes over background. Both the types and concentrations of chemicals that leg chemoreceptors respond to are similar to previous reports of antennular chemoreceptors. Both respond maximally to the following amino acids: L-glutamic acid, hydroxy-L-proline, taurine, betaine, L-aspartic acid, and glycine. These compounds include the free amino acids most abundant in tissues of a number of invertebrate prey for lobsters. This matching would be expected for such a euryphagous predator. Indeed, live prey odors and their tissue extracts are highly stimulatory. Parallel behavioral studies indicate that lobsters respond to extremely dilute live prey odors, containing a total amino nitrogen concentration of approximaely 5×10^{-9} M. Although proteins are not considered to be important chemical stimuli for crustaceans, ultrafiltered hemoglobin (1.25×10^{-1} g/l) and tripeptide glutathione were highly stimulatory to dactyl receptors. Organic acids and sugars were poorly stimulatory for both antennules and legs. Leg thresholds (L-glutamic acid, 5×10^{-7} M; L-alanine and glycine, 5×10^{-6} M) are also similar to antennular thresholds. This contradicts the differentiation of antennular and leg receptors on the basis of thresholds.

Although other response characteristics (latencies, durations, adaptation rates) were not analyzed, the similarities in the studied peripheral receptor responses are striking. The cause of the differential behavioral functions of leg and antennular chemoreceptors, as reported elsewhere, may therefore lie in the neuroanatomical connections of these peripheral receptors.

Inhibition of lysosomal enzyme secretion from dogfish phagocytes inhibits their cytotoxicity to sea urchin eggs. P. DUNHAM, P. ARVAN, S. FALKOW, AND G. WEISSMANN.

Blood phagocytes of the dogfish *Mustelus canis* attack oocytes of the sea urchin *Arbacia punctulata,* first provoking a surrogate fertilization response, and then killing the eggs. To test the hypothesis that secretion of lysosomal contents at zones of phagocyte-egg contact is critical in this model of phagocyte-mediated cell injury, we studied the effects of various agents which modify lysosomal enzyme secretion. When present at concentrations ranging from 0.1 to 5.0 mM, inhibitors of membrane transport inhibited the post-phagocytic secretion of lysosomal β-glucuronidase from dogfish phagocytes induced by zymosan particles: phloretin > ethacrynate > furosemide > SITS >> pyridoxal phosphate > ouabain. The same order of activity was found for the inhibition by these agents of cytotoxicity provoked in *Arbacia* eggs by dogfish phagocytes. Cell activation (fertilization response) and cytotoxicity were quantitated by supravital observation (Nomarski optics) and measurements of enzymes (β-glucuronidase, catalase) released from the agonal oocytes into the suspending medium. The agents themselves neither inhibited the fertilization response of the eggs to ionophore A-23187, nor impaired the viability of the phagocytes (judged by leakage of cytoplasmic lactic dehydrogenase and vital staining with neutral red). Vital staining demonstrated that ethacrynate prevented degranulation of lysosomes into phagocytic vacuoles surrounding zymosan particles, and ethacrynate-treated cells did not degranulate upon contact with *Arbacia* eggs, although attachment was unimpaired. These data suggest not only that a variety of agents primarily known for their capacity to inhibit membrane transport systems can inhibit lysosomal enzyme secretion from phagocytes, but also support the hypothesis that secretion of lysosomal contents mediates activation and killing of target cells in phagocyte-mediated tissue injury.

Characterization of the microfilamentous cytoskeleton of sea urchin coelomocytes. KENNETH T. EDDS.

The cytoskeleton from both the petaloid and filopodial coelomocytes of *Strongylocentrotus droebachiensis* have been isolated and characterized by SDS gel electrophoresis. Pure populations of each form of the coelomocytes were obtained by centrifugation of coelomic fluid on sucrose gradients and washing in an isotonic buffer. The washed cells were lysed with 1%

Triton X-100 in a solution containing 0.125 M NaCl, 3 mM $MgCl_2$, and 10 mM TES, pH 7.5. The unlysed nuclei were shared from the detergent resistant cytoskeletons and pelleted by centrifugation @ $2{,}000 \times g$ for 5 min. The resulting cytoskeleton-rich supernatant was concentrated and samples were prepared for SDS gel electrophoresis. Densitometer scans of the Fast green stained gels revealed that the major component of the cytoskeletons, actin, comprises approximately 80 to 85% of the total cytoskeletal protein. Additional actin-associated proteins appear at 27,000, 57,000, 64,000, and 220,000 daltons. Qualitatively, the petaloid and filopodial cytoskeletons are isolated with identical components; quantitatively, however, the amounts of the 57,000 dalton component are different. The filopodial preparations contained an average of 2.5 times more of the 57,000 dalton component per mole of actin than the petaloid preparations. This increase appears to result from an incorporation of that component into the transforming cytoskeleton and it is presumed to crosslink filaments in bundles and stabilize the three dimensional nature of the cytoskeleton.

Supported by PHS Grant GM-23351.

Behavioral evidence for functional separation of taste and smell in the lobster, Homarus americanus. SUSAN EDER AND JELLE ATEMA.

In an aquatic environment, chemical stimuli will reach all the chemoreceptive appendages of an organism. The existence of different chemoreceptor organs suggests that receptors have different behavioral functions (*e.g.,* taste and smell in aquatic invertebrates). We have studied the responses of the lobster (*Homarus americanus*) to stimulation of various appendages to test the hypothesis of functional separation of chemoreceptor organs in invertebrates.

The antennules, walking legs, maxillipeds, and seizer claw were stimulated by injecting mussel juice into a constant, fine flow of sea water running through tubes attached to each appendage. Stimulation of the antennules resulted in increased activity of all appendages, with a significantly greater frequency of general alert responses than feeding responses. When the walking legs or maxillipeds were stimulated, there was a significantly greater frequency of feeding responses than general alert responses. Seizer claw stimulation resulted in fewer responses than walking leg or maxilliped stimulation, with a trend toward more frequent feeding than alert responses.

These data suggest that there is a functional distinction between the chemoreceptor appendages of the lobster. The walking legs, maxillipeds, and claws appear to elicit similar, stereotyped responses and play a role in feeding. This corresponds to vertebrate taste function. The antennules appear to provide general stimulation and correspond to vertebrate olfaction.

The role of wood decomposition in nitrogen cycling in forest ecosystems. KEITH N. ESHLEMAN, JOHN D. ABER, AND JERRY M. MELILLO.

The influence of decomposing wood on nitrogen dynamics in forest ecosystems was examined in a field study. The following null hypothesis was tested: the nitrogen content of decomposing wood does not increase on an absolute scale during the decomposition process; that is, decomposing wood does not act as a nitrogen sink during any phase of the decomposition process. This hypothesis was tested by studying changes in weight and nitrogen content of decomposing yellow birch (*Betula alleghaniensis*) and paper birch (*Betula papyrigera*) branches and stems lying on or above the forest floor in a deciduous forest ecosystem at the Harvard Forest, Petersham, Massachusetts. Statistical data analysis suggests that decomposing birch wood is not a nitrogen sink at the site studied.

The relationship between original biomass remaining (%) and nitrogen concentration (%) has been shown to be an inverse linear function with high correlation for a variety of plant residues. Birch wood data plotted in this manner showed a regression line slope and correlation coefficient of −48.6 and −0.58, respectively. A plot of the data on logarithmic scales, however, showed a slope and correlation coefficient of −0.56 and −0.78, respectively. The plot of a hypothetical data set for which substrate nitrogen is conserved but no net immobilization occurs on the logarithmic scales shows an inverse linear function.

Inhibition of sea urchin development by low concentrations of the tumor promoter TPA; protection by a protease inhibitor. H. Esumi, J. P. Quigley, and W. Troll.

The tumor promoter 12-0-tetradecanoyl phorbol-13-acetate (TPA), the active component of croton oil, accelerates tumor formation in carcinogen-treated mouse skin. Moreover, TPA inhibits differentiation in a number of tissue culture systems. We now report the inhibition of differentiation of a developing echinoderm, *Arbacia punctulata.* Inhibition is most striking at the blastula stage. At 10^{-9} to 10^{-8} M TPA, hatching of blastula is inhibited and low yield of the plutei result. Above 10^{-7} M TPA the blastula falls apart, resulting in cellular debris without survivors. The protease inhibitor, elastatinal at 5×10^{-4} M, counteracts the TPA inhibition of plutei formation (10^{-9}–10^{-8} M TPA). At 10^{-7} M TPA, elastatinal permitted survival of some moving blastula and sharply reduced the degradation brought on by the high concentrations of TPA.

Proteases are induced by TPA in mouse skin and tissue culture systems. One of the consequences of TPA action is the reprogramming of the genome and it has been suggested that this is accomplished by removing repressor proteins through the enhanced proteolytic activity of the TPA-treated cells.

Using a fluorescent substrate, we measured an elastase-like enzyme in sea urchin eggs and the production of this enzyme was increased following TPA treatment. The catalytic activity of this enzyme was partially to fully inhibited by 5×10^{-4} M elastatinal.

The mechanism of disturbing normal differentiation of arbacia embryos by TPA may involve a reprogrammed genome. We observed an initial increase in cell division after 10^{-9} to 10^{-8} M TPA treatment followed by irregular cell division during the next 4 cleavages. Elastatinal appeared to prevent this, although the effect has not yet been quantitated. The action of TPA as a tumor promoter may be the disturbance of normal development setting up a favorable situation for a chemically or virally modified cell to grow out.

Exogenous ATP interaction with leucine transport—intestine of the toadfish, Opsanus tau. A. Farmanfarmaian, Derek Barkalow, James Bush, and Robin R. Socci.

It is well known that the active transport of amino acids is ultimately dependent upon cellular energy, although the direct bioenergetic coupling mechanism remains controversial. One well promoted view is the so-called "gradient theory" in which cellular energy is transduced into a Na^+–gradient or Na^+–electrochemical gradient which, in some mysterious way, is coupled to a brush border "carrier" protein engaged in the uphill transport of an amino acid or sugar molecule. We have previously presented evidence which does not support the Na^+–gradient as a source of energy for the active transport of sugars and amino acids in the intestine. Approximately a 30 to 70 mM Na^+ solution is required at the membrane, but a Na^+–gradient is not essential. More recently, we have considered the possibility that ATP may be directly coupled to the intestinal amino acid carrier. The transport of L-alanine, glycine and L-leucine is inhibited by 50% or more when 10 mM ATP is added to the luminal medium. The transport system is specifically influenced by ATP in that ATP hydrolysis (Pi production) is stimulated by the transported amino acid. We interpret these observations to mean that the amino acid carrier mechanism can be directly phosphorylated or energized by cellular ATP from the cytosol side to inhibit amino acid efflux and effect its cellular accumulation, or by exogenous ATP to inhibit influx in a contramimicking of the normal cellular ATP action.

Supported in part by the funds from the Center for Coastal and Environmental Studies, Rutgers University, New Brunswick, New Jersey and Busch Funds to the Bureau of Biological Research, Rutgers University.

Fluoride-induced discrete waves in the ventral photoreceptors of Limulus. Alan Fein and D. Wesley Corson.

Illumination of *Limulus* ventral photoreceptors with dim lights results in a response that is composed of discrete waves. These discrete waves are believed to result when a single visual pigment molecule is activated by the absorption of a quantum of light. Discrete waves have also been found to occur spontaneously in the dark at a slow rate. We have found that the addition of 10 mM NaF to the saline (pH 7.0) that bathes *Limulus* ventral photoreceptors results in a dramatic increase in the rate of discrete waves recorded in the dark. These fluoride-induced discrete waves can be observed in both voltage clamped and unclamped photoreceptors. Their time course is similar to the time course of discrete waves elicited by a light stimulus, and they can be adapted by a bright light flash. Therefore, they appear to be similar to the discrete waves evoked by a light stimulus.

Bathing the photoreceptor in saline (pH 7.0) + 10 mM NaF results in a large increase in discrete wave frequency after 15 to 20 min. Returning to fluoride-free saline reverses the effect of the fluoride after 30 to 60 min. Exposure for up to 60 min in 10 mM NaF saline at pH 7.8 does not result in an appreciable increase in discrete wave frequency. Thus, the ability of fluoride to induce discrete waves appears to be pH sensitive.

The distribution in time of the occurrence of fluoride-induced discrete waves is highly dependent on the Ca concentration of the saline. Lowering the Ca concentration from 10 to 1 mM results in a pronounced clustering in time of the fluoride-induced discrete waves.

Finally, the duration of the response to a test flash is reversibly increased upon exposure to 10 mM NaF saline at pH 7.0.

Fertilization of surf clam oocytes: The role of membrane potential and internal pH. TOREN FINKEL AND DON P. WOLF.

A membrane potential of -18 ± 6.6 mV ($N = 20$) inside negative was observed in the unfertilized *Spisula* oocyte. Five seconds after sperm addition, the cell depolarized, reaching a peak within 60 sec. Usually a reversal of membrane polarity occurred, with some cells reaching as much as +20 mV (inside positive). Membrane potentials remained around 0 mV for the next 4 to 5 min and then began to hyperpolarize, reaching a new resting level of −70 mV in 1 to 3 min. In other marine oocytes, rapid membrane depolarization was attributed to increased sodium conductance which, in turn, produced a block to polyspermy. In *Spisula*, oocytes inseminated in low sodium seawater displayed ten times the control levels of polyspermy, suggesting that there is also a sodium-dependent block to polyspermy in this species.

Cytoplasmic activation during fertilization or parthenogenesis may involve changes in pH; therefore, experiments designed to alter intracellular pH were conducted. Agents which may raise internal pH, ammonia and procaine, produced germinal vesicle breakdown (GVB), provided extracellular calcium was present. Conversely, weak organic acids, which should cross membranes in their undissociated form and subsequently lower internal pH, inhibited sperm-induced GVB. The degree of inhibition seen with acetate- or proprionate-seawater was dependent on extracellular pH. If eggs were inseminated in seawater and 2 min later transferred to acetate-seawater, blockage still occurred. Internal pH changes could result from proton efflux. A drop in extracellular pH was recorded in oocyte suspensions for the first 6 min following sperm addition. The response was oocyte-concentration–dependent but did not correlate with the degree of GVB. It remains uncertain whether the acid came directly from fertilized oocytes or arose from sperm-egg interactions.

Our results, therefore, suggest that an electrical block to polyspermy occurs in *Spisula* and that an elevation in intracellular pH is a component of cytoplasmic activation.

Supported by USPHS Grant #HD07638 to D.P.W.

Photopositive behavior of young Limulus. KATHLEEN FRENCH AND SUSAN DOLINER.

Laboratory-raised trilobite larvae and juveniles in the first tailed stage were tested to determine their light intensity preference. Eggs were fertilized *in vitro* or collected within 12 hr of spawning and were kept on a 14-hr light/10-hr dark schedule throughout development.

During daytime hours, animals were taken directly from room light to the experimental set-up (light adapted = LA) or were held in the dark for 30 min before the experiment began (dark adapted = DA). They were then placed in a glass dish with a bright area, a very dim area, and a narrow transitional area. The incident light passed through a heat filter, and the temperatures in bright and dim areas were the same. Some individuals were continuously tracked. In other experiments, eight *Limulus* were started in each of the three areas, and their distribution was observed periodically. The light was on for at least 100 min in each group experiment, with at least one 30-min dark period intervening.

The number of DA trilobites in the bright area increased steadily while the light was on. The LA trilobites congregated in the dim area for the first 15 min of the first light-on period and then increased in number in the bright area while the light was on. Individual trilobites usually remained actively swimming in the bright area once they entered it. The juveniles swam actively during the daytime experiments, but their distribution appeared to be random. When nighttime experiments were done, however, the number of juveniles in the bright area exceeded that in the dim area.

A marked change in the response of *Limulus* to light therefore appears to occur with the first molt after hatching.

This work was supported by a grant from the Rowland Foundation to the Laboratory of Sensory Physiology.

Electronic and dielectric properties of protein-methylglyoxal complexes. PETER R. C. GASCOYNE AND RON PETHIG.

Samples of bovine serum albumin (BSA), casein, collagen and lysozyme have been complexed with methylglyoxal. As shown by Albert Szent-Györgyi, such treated proteins assume a stable brown color. Compared with the untreated proteins, these brown proteins exhibit an interesting range of electronic and dielectric properties, which include a large electron spin resonance signal, a greatly enhanced DC and low frequency conductivity, and a low-frequency dielectric dispersion. These properties have been found to be significantly dependent on the hydration content of the test samples. The ϵ-amino groups of lysine residues have been reductively methylated for casein and BSA, and the amount of free lysine remaining has been determined spectroscopically. A direct relationship appears to exist between the number of free lysine groups and the color and ESR intensity that result after these modified proteins have been reacted with methylglyoxal. From our studies and the theoretical considerations of others, we consider that methylglyoxal forms a Schiff base linkage with the ϵ-amino groups of the lysine residues and that this Schiff base subsequently acts as an electron acceptor in an inter-molecular charge-transfer reaction with the polypeptide backbone of the protein. As a result, the valence band of extended ground-states in the backbone becomes partially desaturated of electronic charge and this effect leads to the electronic and dielectric properties observed experimentally.

This work is sponsored by the National Foundation for Cancer Research.

Heartbeat mechanism in embryonic Limulus. DANIEL GIBSON AND FRED LANG.

We refute the widely cited conclusion of Carlson and Meek (1908, *Am. J. Physiol.,* **21**: 1–10) that the initial heartbeats in *Limulus* embryos are myogenic. We determined the onset of heartbeat to be concomitant with the third embryonic molt (of four), when only the anterior half of the dorsal tubular heart has formed. Observation of heart movement was facilitated by vital staining with neutral red, which enhanced contrast of the heart walls within the coelomic space. Rapid flash (64 μsec) photographs showed that the intermittent beats were synchronous along the formed segments of the heart, as in the adult; early beats were not peristaltic as previously reported. Ultrastructural evidence for innervation at the onset of heartbeat was in the form of axon bundles which we consistently found along the dorsal midline of any lumen-containing heart region. Vesicle-filled terminals forming junctions with the muscle were frequently found with the axon bundles. Intracellular recordings made with floating microelectrodes revealed excitatory postsynaptic potentials, caused by nervous discharge on the muscle. Contractions occurred only when several EPSPs summated to produce depolarizations of 20 mV or more (resting potential −60 mV). Failure of summation was frequent in young hearts, but rise times (10 msec) and general form of depolarization were the same as in older, neurogenic stages. We never observed any of the slow, overshooting spikes typical of myogenic hearts. Our photographic, ultrastructural, and electrophysiological evidence leads us to conclude that embryonic *Limulus* hearts are driven by the cardiac ganglion from the beginning and do not go through an initial myogenic stage.

This work was supported by NIH Grant RO 1 HL18267.

Trail following by Littorina: *washout of polarized information and the point of paradox test.* W. F. Gilly and R. P. Swenson.

Littorina snails can follow underwater mucoid slime trails deposited by members of their own species, and following reliably occurs towards the leading snail. *L. sitkana* is capable of correct following when tested on screen (35μ pores) covered or "backwards" trails (Gilly and Cahalan, 1978, *Sixth Int. Biophysics Congress, Kyoto*), and this suggests following may involve detection of a chemical substance rather than of some physical asymmetry. We hypothesize that slow diffusion of some substance out of the trail establishes a gradient, and that this could be the primary mechanism underlying trail polarity recognition.

Decline in ability of follow as a function of trail age was studied with *L. littorea.* A follower snail was directed to approach an aging trail at right angles, but upon contacting it at time T (trail age) was then allowed 360° in which to turn. At $T \leq 2$ min, correct following occurred with a chance $P = 0.85$ ($N = 24$) and in the wrong direction (away from the leader) at $P = 0.1$. At long T (>30 min), correct following dropped to $P = 0.3$ to 0.4 with the chance of wrong following rising to a similar level ($N = 15$). Between these limits decay of correct following could be characterized by a time constant of 7 min. Snails crossing the trail (assumedly not detecting it) have been excluded from this analysis. P for crossing was constant at about 0.1 until T approached 20 to 30 min; P then rose to about 0.3.

If the substance in the trail disappears at a rate of 63%/7 min, a gradient of 2%/cm must be detectable (mean trail deposition velocity of 5 cm/min and a maximum distal tentacle span of 1 cm). Trail following might thus involve detection of this uphill gradient.

In a different experiment, a second snail followed the trail of a first to a point X and was then removed. A third snail was directed to contact the double trail precisely at X, thus encountering a paradoxical large uphill gradient in the direction opposite to which the trails were laid. Following towards the first leader occurred in 8 of 10 trials. No snails followed in the wrong direction, the two failures turning away from the trail. These observations suggest detection of the anamalous gradient may have occurred, but a more complicated integrative mechanism is probably also involved in decision making.

Temperature conditional luminescence in natural isolates of luminous bacteria. David Goldfarb, Allan Kaster, and Alex Keynan.

The induction and synthesis of the bioluminescent system in luminous bacteria has been shown (in recent years) to be independent of growth under some conditions. It was therefore of interest to find two newly isolated strains (FF and F1), tentatively classed in the genus *Photobacterium,* which were nonluminous at 28°, a temperature at which good growth was nevertheless possible. In cultures of F1 grown at 13°, induction of luminescence occurs at mid-log (O.D. 600 ~ 0.5) peaks at stationary phase, and light emission continues for several days. At 28°, growth is somewhat slower but visible light never develops. At 26° some (10-fold less) light is produced, but after its maximum, drops to zero within 4 hours. In cells grown at 23° and transferred to higher temperatures (>29°), the luminescence decreases after a few minutes. Thus, this temperature effect is a physiological one and is not due to changes in population, which might lead to the selection of dark variants. Comparing the rate of decrease of light emission as a function of temperature, an apparent activation energy of about 10 kcal was obtained over the temperature range 29 to 35°. This suggests that the rate of some key enzymatic reaction is involved in this inactivation rather than protein denaturation. An indication of the nature of this reaction was found when it was shown that luminescence in cells which had become dim following transfer from low to high temperature could be stimulated by the exogenous addition of long chain aldehyde, one of the factors required in the *in vitro* bioluminescent reaction. The experiments indicate that, in at least some strains of luminescent bacteria, the aldehyde forming system is more temperature sensitive than is the luciferase. The phenotypic behavior of these strains is similar to temperature conditional aldehyde mutants isolated by Cline and Hastings, and they could be used in the investigation of the relative induction of luciferase and aldehyde synthesis.

The authors thank J. W. Hastings for his interest and help in this study, and the Max Bogen Foundation for the financial support which enabled this research.

The embryotoxic effect of a commonly used vaginal antiseptic Povidone-Iodine (Betadine®). DORIA R. GORDON, PNINA G. ROSENKRANZ, WILLIAM T. SPECK, AND HERBERT S. ROSENKRANZ.

Earlier we demonstrated the mutagenic and DNA-modifying activities of a number of genitourinary antimicrobial agents including Povidone-Iodine (PVP-I, Betadine®). The activity of PVP-I appears to be related to its ability to iodinate the cytosine residue of DNA. Alteration of the cellular DNA can lead to genetic, carcinogenic and teratogenic consequences. This investigation evaluates the effects on gametes, fertilization and embryonic development of the American sea urchin *Arbacia punctulata* of an over-the-counter (OTC) preparation of PVP-I: Betadine Douche® (BD) used for vaginal antisepsis because the human embryo and gametes may come into contact with this product.

At commonly used levels (1/100), BD caused loss of fertilizability of both sperm and egg. At higher dilutions (1/1000–1/100,000) developmental abnormalities occurred: high proportions (30–70%) of abnormal but viable plutei. Addition of BD during development resulted in arrest at dilutions of 1/100 and the accumulation of large numbers (50–100%) of abnormal plutei when BD was diluted 1/10,000 to 1/100,000. Another OTC preparation of PVP-I (Betadine® Solution) also exhibited embryotoxic properties. Unsubstituted PVP at comparable concentrations had no effect.

The present findings which indicate that PVP-I decreases fertilization and induces developmental abnormalities in the sea urchin, when combined with our previous observations of DNA-modifications, suggest that PVP-I has the potential for causing developmental abnormalities in the human embryo as well. It is suggested that the OTC availability of PVP-I for vaginal antisepsis be regulated.

A nitrogen budget for the marram grass dune community of Cape Cod, Massachusetts. RICHARD H. GRANT.

A preliminary nitrogen budget for the summer months of the marram grass (*Ammophila breviligulata* Fern.) community on the north spit of Great Sippiwissett Marsh (Falmouth, Massachusetts) was formulated to determine the importance of nitrogen fixation in the sand soil system. Major nitrogen inputs for the plant-soil system (to 50 cm depth) were precipitation, nitrogen fixation, and tidal water. Model state variables included: soil organic nitrogen (24 ± 2.4 g N/m^2), shoot nitrogen (4.97 ± 0.61 g N/m^2), rhizome nitrogen (1.15 ± 1.45 g N/m^2), and root nitrogen (0.90 ± 0.63 g N/m^2). Variation in the state variables (mean ± standard error) was due to biomass variation and not nitrogen content. Tidal inputs (unmeasured) are present only for plants at the dune base in the system studied. Precipitation inputs of inorganic [99.2 mg N/(m^2·92 days)], organic [106.4 mg N/(m^2·92 days)], and particulate nitrogen [7.8 mg N/(m^2·92 days)] (Valiela *et al.*, 1978, *Limnol. Oceanogr.*, **23**: 798) add 0.2 g N/(m^2·92 days) to the system. Nitrogen fixation was found in association with the marram grass roots and in the open sand. Root associated nitrogenase activity was estimated at 2.2×10^{-5} g N(C_2H_2)/(m^2·92 days) on excised roots using the theoretical 3:1 ratio of moles acetylene reduced to moles nitrogen fixed. Soil nitrogen fixation by photosynthetic microorganisms was less than one-tenth of the root fixation per square meter. Decomposition (nitrogen recycling) is rapid in the aerobic sand soil, as evidenced by the soil organic matter content of 0.12% (oven dry wt) and the C/N ratio of 34.

Assuming steady state conditions where atmospheric inputs of precipitation and nitrogen fixation equal leaching outputs and the shoot nitrogen approximates the total plant nitrogen requirements, the nitrogen turnover time is 4.8 years. If the nitrogen requirements were supplied by decomposition and the atmospheric inputs, 21% of the soil organic matter (168 g) would be mineralized per year. Nitrogen fixation contributes only 1% of the atmospheric inputs

[0.2 mg N/($m^2 \cdot 92$ days)] and only 0.004% of the estimated nitrogen requirements of the marram grass.

This work was made possible by support from the Jessie Smith Noyes Foundation and the SURDNA Foundation.

Hydrogen formation by marine algae. ELIAS GREENBAUM, DAVID MAUZERALL, AND ROBERT R. L. GUILLARD.

Using a sensitive ZrO_2 electrode, we sought light driven photosynthetic hydrogen production in several marine algae. These were illuminated by a rapid burst of single turnover flashes with systematically varied numbers of flashes within each burst. We measured decrease in H_2 yield per flash as a function of number of flashes within each burst. The reduced yield/flash is interpreted as depletion of a pool of reducing equivalents. The rate of flashing within each burst was 7 Hz, corresponding to a time much longer than the fast turnover time in photosynthetic hydrogen production, 0.1 to 5 msec. Several marine clones of *Chlamydomonas* (0–5, Y, 6–33, 11/35, D) evolved H_2 in light. We estimate that 0 to 5 has a pool of 20 to 30 reducing equivalents, approximately equal to the pool size for the freshwater *Chlamydomonas reinhardtii* and to the number of equivalents in the plastoquinone pool linking the two photosystems of photosynthesis. We hypothesize that the plastoquinone pool is in turn reduced by cellular metabolites. No *Chlamydomonas* clone liberated H_2 in the dark. Neither light nor dark H_2 evolution occurred in the phytoplankters *Platymonas* (Platy 1), *Synechococcus marina* (Syn), *Thalassiosira pseudonana* (clones 3H and 13-1), *Amphidinium carterae,* nor *Chroomonas salina* (3C), but *Oscillatoria* (Sm 24) produced a little H_2 in darkness. Dark H_2 evolution also occurred in freshly collected *Chondrus crispus, Enteromorpha linza, Ulva lactuca, Codium fragilis, Ceramium rubrum,* and *Neoagardhiella baileyi,* but not in *Fucus vesiculosus* nor *Porphyra umbilicalis.* No macroscopic alga studied showed light-driven H_2 evolution. We suggest that in eukaryotes, if the anaerobically induced hydrogenase forms in the choloroplast, H_2 evolution is coupled to light, while if formed outside the chloroplast, H_2 evolution is coupled to metabolites.

Supported by the Experimental Marine Botany Course; Technology Department, Medical Products Division, Union Carbide Corp.; and NSF Grants #BMS74-11747 and OCE78-08858.

Repetitive firing in squid axon membrane as a model for a neuronal oscillator. RITA GUTTMAN AND STEPHEN LEWIS.

Repetitive firing (an infinite train) occurs in space clamped squid axons bathed in low calcium sea water (10 mM $CaCl_2$) when stimulated with a step of suprathreshold current. The repetitive firing could be annihilated by applying a short pulse of the proper magnitude and at the proper phase. This annihilation was complete (involved no subthreshold oscillation) if the pulse displaced the trajectory to the immediate area of the stable singular point, causing it to locate there very quickly.

In other cases, when the applied pulse was of different magnitude and at a different phase, there occurred small subthreshold oscillations as the trajectory spiraled into the stable singular point. If the perturbation was of a magnitude and at a phase just adequate to enter the domain of attraction of the stable singular point, the subthreshold oscillations slowly diminished and finally died out. At other magnitudes and at other phases, the perturbation resulted in oscillations which grew in size until they finally exploded into spikes; the trajectory apparently spiraled into the stable limit cycle of repetitive firing. When reentry into the stable limit cycle of repetitive firing occurred due to a perturbation, the new spikes, though of the same frequency as the original, occurred at a different phase compared to the extrapolated original train of spikes. Thus, repetitive firing in a patch of squid axon membrane pursues a limit cycle oscillation. If our experimental perturbation is analogous to a synaptic potential, it is seen that a given synaptic potential may be more or less effective when affecting a repetitively firing axon, depending upon where in the phase of the limit cycle the synaptic potential occurs.

These experiments constitute a map of the sensitivity versus the phase of the stable limit cycle of a repetitively firing, excitable membrane.

Aided by NIH Grant 5 R01 NS 12272-03.

A dual effect of diamide, a thiol oxidizing agent, on the photoresponse of the Limulus *ventral eye.* MENACHEM HANANI AND ALAN FEIN.

Biochemical studies on vertebrate rhodopsin have shown that the molecule contains several thiol groups. Some of these groups may be involved in the molecular transformations that follow the absorption of light, but the physiological significance of these findings is not known. We studied the effects of diamide, a thiol oxidizing agent, on the ventral photoreceptors of *Limulus* using electrophysiological techniques. Diamide is known to cross cell membranes and its main action is to transform reduced glutathione to oxidized form. In a typical experiment, addition of 10^{-3} M diamide to the saline caused the following sequence of events. The membrane potential depolarized slightly (2–4 mV) and there was an increase in the response to light. After 3 to 5 minutes in diamide, there was a large decrease in the response to light, which was reversed only after normal saline was returned to the chamber. These results were obtained with intracellular voltage or current measurements. In parallel with the desensitization, there was a decrease in the amplitude of discrete waves which recovered together with the photoresponse. In some cases there was an initial increase in the amplitude and frequency of the discrete waves after diamide application. A decrease in the photoresponse after adapting with bright lights is a well known property of photoreceptors. The desensitizing action of diamide may be produced by a process similar to light adaptation. To test this possibility, we compared response wave-forms during light adaptation with those in diamide. The responses in diamide were much more prolonged and had longer latencies compared to equally desensitized responses in the light-adapted state. This suggests different mechanisms for light adaptation and the action of diamide. The dual effect of diamide suggests that it acts at two different sites, each being oxidized at a different diamide concentration.

Ultrastructure and physiology of gap junctions of an amphibian embryo, effects of CO_2. R. B. HANNA, D. C. SPRAY, P. G. MODEL, A. L. HARRIS, AND M. V. L. BENNETT.

Pairs of blastomeres were isolated from 16-256 cell blastulae of the axolotl *Ambystoma mexicanum.* Alternatively, pairs were formed from single cells. Microelectrode measurements show that these pairs are electrotonically coupled, and the dye Lucifer Yellow passes between them. Bathing in saline equilibrated with 100% CO_2 causes a rapid (within 60 sec) and reversible decrease in junctional conductance. Blastulae at similar stages were fixed for electron microscopy. Thin sections show infrequent gap junctions of typical septalaminar appearance. Smaller gap junctions appear more frequently in freeze fracture replicas. These junctions are characterized by aggregates of P face particles and E face pits and a narrowing of the intercellular cleft. The particles are approximately 9.5 nm in dimeter, which is larger than most particles in the neighboring nonjunctional membrane. Replicas of gap junctions of high resistance were obtained from tissue bathed in CO_2 saline for 10 min. Particles in these junctions appear somewhat more distinct and more regularly arranged, but even small regions of hexagonal packing are rare. Particle counts showed a 20% increase in density in high as compared to low resistance junctions, but the validity of the measurement is questionable because of the greater distinctness of the particles in the high resistance state. Gap junctions from tissue bathed in normal saline for 10 min after CO_2 exposure were indistinguishable from high resistance junctions, although junctional conductance should have recovered to normal levels. Further study of this tissue should aid in the evaluation of morphological correlates of functional state.

Energy dispersive X-ray analysis of cataractous lenses. C. HARDING, S. SUSAN, B. WORGUL, A. SOLWAY, AND M. MASER.

Fresh human cataractous lenses were gently blotted, cut into quarters, and dried in a desiccator. During the drying process, the lens fragments shrank, and in some cases, blebs of material appeared on the cut surfaces. Some lenses were gently compressed, and this seemed to increase the amount of bleb material. The specimens were carbon-coated, examined with a scanning electron microscope (Philips 501), and analyzed by means of energy dispersive X-ray analysis (EDAX 711) and in the M.B.L., E.-M. Lab. The spectra showed a relatively high phosphorus and low sulfur content in the bleb material, and the opposite (low phosphorus, high sulfur) in the surrounding fiber cells. We suggest that the "blebs" may represent material from the "water clefts" characteristic of human cortical cataracts.

Supported in part by NIH Grant EYO 1874, Energy contract, EY-76-S-02-2401, and a grant from Research to Prevent Blindness, Inc.

Analysis of acrosomal and nuclear proteins in Thyone *sperm.* LOUIS HERLANDS AND LEWIS G. TILNEY.

The sperm of *Thyone briareus,* as well as the sperm of many marine invertebrates, contain somatic-like histones as judged by a variety of different electrophoretic techniques. EM studies show that the nucleosomes in *Thyone* are almost exclusively organized into highly ordered 300 Å structures. However, when sperm heads are acid extracted and proteins analyzed on acid/urea gels, there is an additional component whose electrophoretic mobility is much greater than any of the core histones. Several other types of sperm which have prominant acrosomes, such as *Mytilus edulis,* also have similar fast moving protamine-like components in addition to histones. Since extremely basic protamine-like proteins have been found in the acrosome of other invertebrates (Das *et al.*, 1967, *J. Cell Biol.,* **35**: 455–458) it might be thought that this fast moving protein is acrosomal in origin. We have used procedures previously described (Tilney, 1976, *J. Cell Biol.,* **69**: 73–89) for fractionating echinoderm sperm into its 3 structural domains: tail/nucleus/acrosome combined with a method for running SDS gels on samples which contain large amounts of viscous DNA (Herlands, unpublished). This technique permits us to localize this protamine-like component to the nucleus. On the other hand, a low molecular weight acrosomal protein which comigrates with histone H4 has been shown *not* to be a nuclear contaminant. This protein's presumed role in association with actin has not yet been determined. In this simplified sperm system, we are now in the process of determining what non-histone structural proteins are present, how they interact with DNA, and how nucleosomes pack to give higher order structures.

Aided by USPHS Grant GM 00265 and by a John Courtney Murray Fellowship to L.H.

The ordered fine structure of neuronal cytoplasm in Loligo *and* Hermissenda. ALAN J. HODGE AND W. J. ADELMAN, JR.

Thin transverse projections radiating from neurofilaments and microtubules are commonly observed in electron microscopy of thin sections of neural tissue. These projections appear as bridges running between microtubules, between neurofilaments, and between microtubules and neurofilaments. Bridges have also been observed ending on the neurolemma and on mitochondrial surfaces.

We utilized improved fixation methods and thicker sections (0.1–0.3 μm) for stereoscopic study of the fine structure of neuronal cytoplasm, primarily in the giant axons of *Loligo pealei* and the CNS of *Hermissenda crassicornis.* Improved fixation was obtained when the initial glutaraldehyde (2–4%) phosphate buffered fixative (pH 7.2) contained EGTA (usually 5 mM) and Mg^{++} (1–5 mM) with the tonicity adjusted with sucrose to about 1100 osmoles. Subsequent fixation and dehydration was conventional (OsO_4, ethanol, Epon 812), except that *en bloc* heavy metal staining was often employed. Sections were often further stained with uranyl acetate and/or lead citrate before examination in a TEM (Philips 300).

Despite the loss of resolution resulting from uncorrected chromatic aberration when observing thick sections, useful details of neuronal structure were obtained, especially in small axons of *Hermissenda.* Stereoscopic examination of neuronal cytoplasm revealed an extensive and apparently highly ordered network of thin (2–5 nm) transverse elements oriented roughly perpendicular to the fiber axis. These appeared to connect the longitudinally oriented neurofilaments and microtubules in a highly ordered three-dimensional lattice structure or gel intimately connected to the neurolemmal surface.

Squid giant axons were fixed by a technique involving initial cannulation of a length of axon followed by slow withdrawal of the cannula with simultaneous release of fixative internally. These fixed axons contained many microtubules and neurofilaments, all oriented parallel to the longitudinal axis and showing a well developed cross-bridge network. The highly ordered cytoplasmic lattice in both *Loligo* and *Hermissenda* neurons appears to be equivalent to the microtrabecular system described by Porter and colleagues and may represent the structural framework presumably required for transport mechanisms such as those proposed by Schmitt for transmitter substances and enzymes.

This research was supported by the Intramural Research Program, NINCDS, NIH.

Metabolic transformation of nicotinamide in oocytes of Spisula solidissima. SABURO HORIUCHI, L. O. BURZIO, AND S. S. KOIDE.

Nicotinamide blocks germinal vesicle breakdown (GVBD) of *Spisula* oocytes induced by KCl or insemination, and inhibits polar body formation. This vitamin also blocks replication of fertilized eggs of *Arbacia punctulata*. The present study was undertaken to establish the chemical nature of the nicotinamide incorporated by *Spisula* oocytes.

Washed *Spisula* oocytes suspended in ASW were incubated with 25 μCi of ^{14}C-nicotinamide at 25°C. Samples were removed for analysis at 0, 3, 10, and 30 min. The oocytes were washed twice with ASW and extracted with 2% perchloric acid. The supernatant was separated by centrifugation and neutralized with 1 N KOH. The precipitate was removed by centrifugation and radioactivity measured with a liquid scintillation counter.

More than 95% of the radioactivity was found in the perchloric acid fraction. The rate of uptake of nicotinamide by the oocytes was the same in the presence of 70 mM KCl in ASW. The radioactive material in the perchloric acid extract was purified on a Dowex 1-2× (200–400 mesh) column and analyzed by polyethyleneimine cellulose thin layer chromatography and by DEAE-cellulose paper chromatography. The major product identified in all samples analyzed was NAD. Other metabolites and nicotinamide were present in trace amounts. The present results suggest that nicotinamide taken up by *Spisula* oocytes is transformed immediately to NAD and supports the thesis that the action of nicotinamide is mediated as NAD.

Supported in part by a grant from The General Service Foundation.

Isolation of an inhibitory fragment from the aggregation factor complex of the marine sponge, Microciona prolifera. TOM HUMPHREYS, WILLIAM W. CRAWFORD, AND A. J. ZUKOWSKI.

When pure aggregation factor from *Microciona prolifera* is heated in the presence of 0.001 M Ca^{++} to 80° for 20 min, an inhibitor of the factor is released from the inactivated complex. Heating at 45° to 65°C inactivates the factor, but does not produce inhibitory materials. Fractionation of heated preparations on a 3000 Å pore size microscope glass bead column showed that the complex is reduced to smaller subunits as the heat treatment generates the inhibitory fragment. Conditions for assaying the inhibitor were studied to maximize sensitivity. One unit of inhibitor was defined as the amount required to produce significant inhibition of the aggregation caused by 4 units of factor. As would be expected from second order reaction kinetics, the assay of inhibitor is strongly dependent on the factor concentration. Doubling the factor concentration more than doubles the inhibitor required to inhibit it fully. Under the standard assay conditions, 16 units of heated factor produced one unit of inhibitor. Inhibitor and factor can be mixed in proportions sufficient to virtually eliminate both factor and inhibitory activity. Such a mixture does not separate into factor and inhibitor when passed over a 3000 Å micropore glass bead column capable of fractionating the two components. The factor and inhibitor appear to bind stoichometrically and strongly to saturable sites on the opposite component. Less than 1% of heat treated factor elutes from a 233 Å micropore glass column with the inhibitor peak. The inhibitor appears to be a minor component of the aggregation factor complex and may represent one of its active sites.

Production and quality of Aufwuchs *as determined by grazing of* Littorina littorea. R. DOUGLAS HUNTER AND W. D. RUSSELL-HUNTER.

The scum-flora or *Aufwuchs* on rock and similar surfaces provides pasturage for a variety of grazing invertebrates. Both productivity and diversity in the *Aufwuchs* crop could obviously be affected by the intensity of grazing. Cage experiments of summer, 1978, tested different densities of the shore snail, *Littorina littorea,* grazing on *Aufwuchs* grown on glass slides mounted in plastic cages near mean low water mark at Nobska Point. Two herbivore densities, low (126 snails/m^2) and high (315 snails/m^2), were tested. Snails were excluded from control cages.

In terms of mg dry weight/dm^2 of *Aufwuchs,* controls moved from 19.5 (day 15) to 314.0 (day 49), while both levels of grazed slides changed only slightly from 5.4 to 9.3. In terms of μg organic carbon/dm^2 over the same period, controls changed from 1612.0 to 15,715.1, low density from 808.2 to 470.4 and high density grazed slides from 480.7 to 454.6. Turning to quality of the *Aufwuchs,* μg carbon/mg dry weight averaged 44.4 for controls, 68.5 and 59.1 for low and high grazed slides, respectively. Perhaps most significantly, the C:N ratio of the *Aufwuchs* from day 6 to day 24 increased from 7.1 to 16.7 for controls but declined in grazed slides from 10.1 to 7.1 for low, and from 8.0 to 4.9 for high density. By day 31, both algal abundance and richness had decreased markedly from controls (102,250 cells/cm^2 and 25 forms) with low grazing (32,012 and 11) and high grazing (28,036 and 10). By any measure, species diversity was reduced.

In a further series of cages with grazer densities from 13/m^2 to 504/m^2, production as either dry weight or carbon/area decreased with increasing density but quality as carbon/mg dry weight apparently increased.

Grazing on *Aufwuchs* by *Littorina* at around natural densities reduces standing crop, productivity and diversity, but increases nutritional quality at some intensities.

Reversible changes in binding of glucose-6-phosphate dehydrogenase to a granular fraction during meiosis in Spisula *eggs.* ICHIO II.

The activity of glucose-6-phosphate dehydrogenase (GPDH) in 0.75 M mannitol homogenate of *Spisula* eggs does not change upon fertilization, nor during early developmental stages (until 4 cell stage). But that in the 100,000 × *g* supernatant increases after fertilization and attains a maximum at the first meiotic metaphase. It then decreases, and almost no activity was observed after the meiosis in the supernatant. This change of activity in the supernatant is accompanied with a mirror-image change of activity in the precipitate, which means a reversible release of GPDH from a granular fraction. The pattern of change of the activity is reproducible, but the extent of released GPDH activity was somewhat different between batches of eggs. The maximum percentage of released GPDH was in a range of 20 to 50% of that in the total homogenate. This reversible release of GPDH from a granular fraction was also observed in *Spisula* eggs activated with KCl. Most of GPDH localizes in the 3,000 × *g* precipitate or in the isolated cortex of unfertilized eggs, and the activities in those fractions fluctuate during meiosis, similarly to the 100,000 × *g* precipitate.

Either NADP$^+$ or NADPH (0.1 mM) releases GPDH completely from the granular fraction of unfertilized eggs *in vitro.* NAD$^+$ (1 mM) does not release GPDH at all. G-6-P (1 mM) has only a slight releasing effect. The concentrations releasing half the enzymes in the granular fraction were 2.5 μM for NADPH and 9 μM for NADP$^+$.

These results suggest a dynamic role of GPDH during meiosis and that the reported increase of NADP(H) concentration after fertilization is responsible for the release of GPDH from a granular fraction, presumably the cortex. The mechanism of recombination remains to be elucidated.

Supported by NIH Grant to Dr. L. I. Rebhun.

Tracking normal and blindfolded Limulus *in the ocean by means of acoustic telemetry.* LEONARD C. IRELAND AND ROBERT B. BARLOW, JR.

Fourteen horseshoe crabs (*Limulus polyphemus*), seven with their lateral and median eyes covered with opaque epoxy, were released individually during daylight hours within a shallow cove near Woods Hole, Mass. All releases were made at a single location, 90 m from shore near the end of the cove and in 2.7 m of water. Previous work shows that wave surge is an important orientational cue for *Limulus.* Wave heights during our tests were 3 to 10 cm, measured trough to crest. A diver releasing neutrally buoyant particles near the bottom at the release site was unable to detect any surge under these conditions. Each crab was fitted with an acoustic transmitter which emitted pulses of sound (50 kHz) at a rate of 0.5 to 2.0/sec. Contact with each crab was maintained by investigators following in a small boat equipped with directional receiving equipment. Position over time was determined from compass bearings from the boat to points on shore. Bearings were taken each 5 to 10 min.

The animals were tracked for periods of 1.8 to 4.0 hr over distances of 0.3 to 0.6 km. Normal crabs displayed travel paths which approximate straight lines, and they left the cove. Blindfolded crabs tended to travel parallel to the shore and remained in the cove. Also, the travel paths of blindfolded *Limulus,* while by no means indicating that these animals were completely disoriented, were more irregular than those of normal crabs. These results show that, under surge-free conditions, covering the lateral and median eyes produces changes in orientation.

Supported by the American Philosophical Society and NIH Grant EY-00667.

The effect of nitrogen on the decomposition of Spartina patens. CYNTHIA L. JENSEN, JERRY M. MELILLO, JOHN D. ABER.

The influence of nitrogen on the decomposition rate of *Spartina patens* leaves and stems was examined in a laboratory incubation study. Two null hypotheses were tested: first, that initial nitrogen content of *Spartina* litter does not influence its rate of decomposition; and secondly, that the amount of dissolved nitrogen in the environment surrounding the decomposing *Spartina* will not influence its rate of decomposition.

To test the first hypothesis, a laboratory decomposition experiment was performed using dead standing *Spartina patens* grown in the fertilized plots at Great Sippewissett Marsh. The plots represented three levels of nitrogen fertilization. The grass was decomposed in half-strength sea water under aerobic conditions and replicates were periodically harvested and analyzed for weight loss and total nitrogen content by the macro-Kjeldahl method. To test the second hypothesis, a similar experiment was run using grass samples grown in an unfertilized plot. The decomposing tissue was subjected to one of three incubation treatments: half-strength sea water with either no, low (10 ppm), or high (100 ppm) ammonium enrichment.

The results of the first experiment showed that rate of decomposition is positively correlated with initial nitrogen concentration. The results of the second experiment showed that enrichment with a high level of ammonium produced a significant increase in the decomposition rate. The data were further analyzed by plotting the percent original biomass remaining against the percent nitrogen for each grass type and/or treatment. It was found that the decomposition process could be described as an inverse linear function with correlation coefficients ranging −0.85 to −0.99. Similar results have been found for a wide range of decomposing tissues.

Stimulation of DNA synthesis in vitro by a factor present in nascent Arbacia *and* Spisula *zygotes.* RAMON NARANJO JIMENEZ, ALBERT GROSSMAN, GARY ROSMAN, AND SADAYUKI INOUE.

Factors present in the soluble fraction of nascent embryos of *Arbacia punctulata* (30 min) and *Spisula solidissima* (60 min) were partially purified and shown to stimulate labeled thymidine 5′-triphosphate (TTP-methyl ^3H) incorporation into DNA *in vitro.* The incorporating system contained: buffer mix (pH 6.8 and 7.4); deoxynucleotide triphosphates with limiting amounts of labeled TTP (no cold TTP); nuclei isolated from fertilized and unfertilized eggs from *Arbacia* and *Spisula,* or mouse liver; and the presence or absence of partially purified activator. Following incubation for 90 min at 25°, the reaction was terminated by addition of 0.5 N $HClO_4$. The precipitate was washed with 0.2 N $HClO_4$ and then heated for 30 min at 90° in 10% NaCl. After filtering, the filtrate was precipitated with cold alcohol, collected on a Millipore filter, and counted. This extensive procedure was employed to remove both free and non-covalently bound TTP-^3H.

Nuclei from unfertilized sea urchin or clam eggs incorporated 10 to 50 times more TTP-^3H in the presence of homologous activator than in its absence. Similar results were obtained with heterologous systems in which activator isolated from the clam was incubated with nuclei from sea urchin. Furthermore, both the activator from the sea urchin or clam stimulated TTP-^3H

incorporation in nuclei from mouse liver. The extent of TTP-^{3}H incorporation was proportional to the concentration of activator used. To demonstrate that the radioactivity incorporated was present in DNA, the labeled material collected on Millipore filters was extracted with phenol. Radioactivity in aqueous solutions of this material was labile in the presence of DNAase. These data suggest that DNA synthesis may be regulated by a factor common to many species. It is possible that the untimely appearance of such a factor may modify the cell growth cycle, perhaps leading to tumorigenesis.

Central control of swimming in Aplysia brasiliana. BEHRUS JAHAN-PARWAR AND DEBBIE L. MILLER.

Swimming in *Aplysia brasiliana* is performed by rhythmic flapping of the parapodia, which are lateral extensions of the foot. Opening and closing waves are produced synchronously from anterior to posterior in both parapodia. Our goal was to determine the roles of the individual ganglia in the control of swimming. The CNS consists of a cerebral ganglion which is joined bilaterally with cerebral-pedal (C-P) and cerebral-pleural (C-PL) connectives to two pedal and two pleural ganglia, respectively. The pedal and pleural ganglia are joined ipsilaterally by pleural-pedal (PL-P) connectives. The two pedal ganglia are connected by the interpedal commissure (P-P). The abdominal and buccal ganglia are not involved in swimming.

A systematic series of lesions of nerves and connectives was performed; the CNS was surgically exposed, nerves or connectives severed, the incision sutured, then swimming observed and filmed. Chronic sleeve electrodes were placed on the proximal cut ends of parapodial nerves. Reversible sucrose perfusion lesions were used instead of cutting connectives in some cases. Lesions and results are as follow: bilateral C-P + C-PL, swimming obliterated; bilateral C-PL, normal swimming; bilateral C-P, swimming obliterated; cut P-P, swimming with parapodia out of synchrony; bilateral PL-P, swimming at reduced speed. Unilateral C-P + PL-P, normal swimming; followed by cutting P-P in same animal: swimming in intact side, lesioned side dormant except for intermittent normal single strokes. Cutting parapodial nerves in one side obliterated swimming in that side. Recording from proximal cut ends of parapodial nerves in one side showed bursting units correlating with ipsilateral parapodial flapping.

The following conclusions may be drawn: first, the motor program for swimming is generated in the CNS; secondly, the cerebral ganglion is the command center for swimming; thirdly, the control center for each parapodium resides in the ipsilateral pedal ganglion; fourthly, command information is carried to the pedal ganglion via the C-P; fifthly, bilateral flapping is coordinated via the P-P; and lastly, the pleural ganglion has a modulatory function.

Supported by a Summer Junior Research Fellowship from the Worcester Foundation for Experimental Biology to DLM, PHS Grants Ns 12483, BNS 77-24174, and a grant from the Grass Foundation.

Activity of microorganisms on surfaces in saltwater. DAVID KIRCHMAN AND RALPH MITCHELL.

Bacteria associated with particles were studied at Little Sippewissett, near Woods Hole, Massachusetts. The area includes a typical *Spartina* saltmarsh and an adjacent brackish water marsh having a salinity of 9‰. Water samples from both marshes were incubated with uniformly labelled [^{14}C]glucose. Subsamples were filtered with Nuclepore filters or Nitex screens of various pore sizes. Approximately 60% of total glucose uptake in the brackish water marsh was due to size fractions larger than 1 μm. In contrast, size fractions larger than 1 μm in the saltmarsh accounted for less than 20% of total glucose uptake. The number of free bacteria and bacteria associated with particles was determined with acridine orange staining and epifluorescence microscopy. In both marshes, less than 10% of the total bacterial populations was present on particles. In the brackish water marsh, the small number of bacteria associated with particles accounted for the majority of glucose uptake. In contrast, free bacteria in the saltmarsh contributed nearly all of total microheterotrophic activity. Apparently it is possible for a small number of microorganisms residing on surfaces in water to account for a major portion of the microbial metabolic activity in the water.

This work was undertaken while one of us (D.K.) was enrolled in the Marine Ecology Course, Marine Biological Laboratory. Contact No. N00014-76-C-0042 between the Office of Naval Research and Harvard University provided partial support.

Segments of sponge aggregation factor which bind specifically to homotypic cells. JAMES E. JUMBLATT, VERENA SCHLUP, AND MAX M. BURGER.

Aggregation factor (AF) isolated from the marine sponge *Microciona prolifera* is a large (2×10^7 dalton) protein-polysaccharide complex which promotes species-specific reaggregation of *Microciona* cells. We have shown previously that iodine-125 labeled AF binds specifically to homotypic cells, although the chemical basis for this specificity is unknown. As a step toward the isolation and characterization of the active site(s) of the AF molecule, iodine-125 labeled AF was dissociated into subunits and proteolytic fragments which retain binding specificity for *Microciona* cells.

Treatment of radioiodinated AF with 5 M urea, 40 mM EDTA at 80° C for 4 hr produced three classes of labeled fragments (peaks I, II, and III) which could be separated by gel filtration on Sepharose 4B. Peak I, which eluted in the void volume, contained less than 10% of the total radioactivity and was presumably enriched in the EDTA resistant "ring" elements of the AF molecule described by Humphreys. Peak II, which contained most of the radioactive subunits, eluted with a V_e/V_o of 0.63. Peak III coincided with the included volume of the column. Radioactive material in peaks I and II was found to bind to chemically dissociated *Microciona* cells with an efficiency comparable to untreated AF. Peak III material, which failed to bind to cells, consisted of very small peptides and free isotope released during dissociation. These data suggested that the "arm" subunits, and possibly the ring element of the AF molecule, interact directly with the cell surface.

Labeled AF treated by the above procedure was digested with 1 mg/ml trypsin for 12 hr at 22° C and electrophoresed on 8% polyacrylamide/SDS gels. A tryptic fragment of 30 to 34,000 MW, which could be eluted from the gel and dialyzed against urea and CMF seawater to remove SDS, was found to bind efficiently to *Microciona* cells. Binding assays performed on cells from other sponge species demonstrated that species-specific binding was retained by all fragments, although somewhat reduced with decreasing fragment size. Whether the 30K fragment contains the primary AF site for cell binding remains to be demonstrated. Such fragments should be, in any case, useful probes for AF function and for further analysis of the surface receptor.

This work supported by the Swiss National Science Foundation.

Inhibition of oocyte maturation by nicotinamide in the surf clam, Spisula solidissima, *and in starfish,* Asterias amurensis *and* A. pectinifera. SUMI L. KOIDE, K. SANO, T. KISHIMOTO, H. KANATANI, AND S. S. KOIDE.

Nicotinamide at concentrations of 1.4 mM or greater prevents germinal vesicle breakdown (GVBD) of *Spisula* oocytes induced by insemination. Partial suppression is observed with nicotinamide at concentrations of 0.34 and 0.68 mM. The drug is effective when added 1 to 3 min after insemination, and is ineffective when added 5 min after insemination. Nicotinamide at concentrations of 3.4 mM or higher prevents GVBD-induced with 70 mM KCl. The drug delays the onset of oocyte activation, rather than causing a complete block. Nicotinamide also prevents polar body formation and cleavage of fertilized oocytes at a concentration of 0.26 mM or higher. The inhibitory effects of nicotinamide on GVBD and polar body formation are reversible. Nicotinic acid at 19.7 mM and thymidine at 50 mM did not influence GVBD or polar body formation induced by insemination or KCl.

1-methyladenine (1-MeAde) is a meiotic-inducing hormone isolated from starfish and plays a role in oocyte maturation and gamete shedding. It induces the formation of a cytoplasmic factor designated as the "maturation promoting factor" (MPF). This factor induces GVBD when microinjected into oocytes.

Nicotinamide at 3.3 mM or higher concentrations inhibits oocyte maturation induced by 1-MeAde in *A. amurensis.* The degree of inhibition by nicotinamide varies with the concentration of 1-MeAde. The block is more pronounced when lower concentrations of 1-MeAde are used, *e.g.*, 10^{-7} M compared to 10^{-6} M. Nicotinamide is effective in blocking GVBD when added prior to and within 7 min after the addition of 1-MeAde. Nicotinamide at 3.3 mM and higher concentrations prevents polar body formation. The block induced by nicotinamide is reversible, and GVBD induced with dithiothreitol is also suppressed by nicotinamide. Furthermore, nicotinamide blocks GVBD induced by microinjection of cytoplasmic MPF into oocytes of *A. pectinifera.* The block induced by nicotinamide is reversible. The present results sugggest that nicotinamide may act by preventing the influence of cytoplasmic MPF on germinal vesicles rather than directly affecting the action of 1-MeAde.

Supported in part by a grant from The General Service Foundation.

The effect of secondary plant substances on grazing of some salt marsh detritivores. L. KOUMJIAN AND I. VALIELA.

Decomposition and energy flow in an ecosystem is dependent on the grazing activity of detritivores. In turn, detritivore ingestion is dependent on the availability and palatability of the detritus. A method that isolates palatability, independent of texture and color of the food source, was used to study grazing preferences of some salt marsh detritivores. Grazing experiments using agar suspensions of the marsh grass, *Spartina alterniflora*, in seawater established that the talitridean amphipod, *Orchestia grillus,* and the snail, *Melampus bidentatus* Say, prefer *S. alterniflora* detritus to live *S. alterniflora.* A third species, the snail, *Littorina littorea* Linn., demonstrated a preference for live over detrital *S. alterniflora.* Grazing activity of the two detritivores, *O. grillus* and *M. bidentatus*, was inhibited in the presence of the secondary plant substances, ferulic acid and p-coumaric acid, in concentrations 3, 5, and 10 times above natural concentrations of 9-month old detritus. Natural concentrations of ferulic acid and p-coumaric acid are 10 mg/g *S. alterniflora* detritus and 3 mg/g *S. alterniflora* detritus, respectively, in the 9-month old detritus.

Fluorescent antibody labelling of Microciona prolifera *aggregation factor and its baseplate component.* W. J. KUHNS, S. BRAMSON, J. JUMBLATT, W. BURKART, AND M. BURGER.

In our earlier studies at this institution, the specificity of goat or rabbit anti-*Microciona prolifera* aggregation factor (AF) for its antigen could be demonstrated based upon inhibition, by derived Fab fragments, of reaggregation of mechanically dissociated cells. Similarly, anti-baseplate (BP) interfered with restitution of hypotonically shocked *Microciona* cells to which BP had been added. In the present experiments, rabbit antisera prepared against highly purified *M. prolifera* AF and BP were utilized in conjunction with goat anti-rabbit fluorescein (FITC) labelled globulin to stain: first, cover slip preparations of mechanically dissociated *Microciona* cell suspensions, secondly, chemically dissociated *Microciona* cells and thirdly, specific *Microciona* cell types which had been enriched using Ficoll gradients. Control slides were prepared using pre-immune sera in conjunction with fluorescein labelled antiserum and labelled antiserum alone. Cells were examined microscopically at low and high power using phase and fluorescence optics. Based upon specific immunofluorescence AF was found to be localized peripherally on most adult sponge cells, including archeocytes, choanocytes, grey cells and rhabdiferous cells, and intercellularly in sponge larvae. If specific anti-AF antibodies were first removed by absorption with purified AF and the centrifuged supernatant used for staining, no peripheral cellular fluorescence was seen on cells. BP appeared to be present on most cells as judged by immunofluorescence, but showed less bright, and at times discontinuous staining of the cell surface, in comparison with AF. Grey cells were typified by yellowish speckled or homogeneous autofluorescence; this may prove useful as a specific cell marker. Differences in the intensity of specific immunofluorescent staining of mechanically and chemically dissociated cells support previous evidence for the Ca^{++} and Mg^{++} dependence of sponge cell-associated AF.

These techniques offer an experimental model for studies of selective cell adhesion and cell differentiation.

Comparative electrophysiological study on the oocyte membrane "neuro-receptors". K. KUSANO.

A possible presence of "neuro-receptors" which are associated with membrane ionic channels was studied comparatively on the ovarian oocytes of the following animals: sand dollar, *Echinarachnius parma;* brittle star, *Ophioderma brevispina;* squid, *Loligo pealii;* and killifish, *Fundulus heteroclitus.* Sand dollar eggs were dechorionated either mechanically or by trypsin. Both defolliculated and untreated brittle star oocytes were examined. The tissues covering the squid and killifish oocytes were removed manually. Acetylcholine (ACh), dopamine (DA), L-epinephrine (L-EP), and serotonin (5-HT) were bath-applied in various concentrations while monitoring the membrane electrical responses (changes in membrane potential, conductance and current flow). The following results were obtained when 5×10^{-5} M of above agents were applied. Sand dollar and brittle star oocytes responded to DA and 5-HT by depolarization and less convincingly to L-EP. These oocytes did not show the response to ACh. The squid oocytes responded to DA, 5-HT, and L-EP by a small depolarization, but the response to ACh was not detected. Killifish oocytes responded to ACh and 5-HT mostly by depolarization, and less clearly to L-EP. However, in a few oocytes hyperpolarizing 5-HT and ACh responses were observed in August. No response to DA was observed. These responses were, in general, associated with an increase in membrane conductance. However, in the echinoderm oocytes a high K-permeability near the resting potential and the presence of depolarizing K-inactivation masked membrane conductance increase by DA but showed, instead, a decrease in conductance in sea water.

Supported by PHS. NS-12275.

Algal species richness and taxonomic clustering in an extreme, stable environment, the Atlantic white cedar swamp. AIMLEE D. LADERMAN.

This study aims to determine if the composition of the *Chamaecyparis thyoides* swamp algal community is dictated by narrow fundamental niches (indicating speciation within the swamps), or by an inability to compete in non-swamp conditions despite a broad fundamental niche. Species richness in the undisturbed cedar swamp is high for a freshwater community. Swamps contain a selectively depauperate fauna: entire taxa are absent (*e.g., Cyanophyta, Rhodophyta, Charaphyceae*). Most families are represented by but one genus, but one family shows high diversity and evidence of radiation (*e.g., Desmidiaceae*). In nature, some swamp species are cosmopolitan; some are restricted to the primary site; others exist in only some of the study sites. Observed algal fundamental niches cover a range of types: first, narrow, highly restricted; secondly, wide, of universal capacity; and thirdly, selective (these yielding clues to true fundamental niche parameters).

Sodium currents through the membrane of squid giant axons. D. LANDOWNE AND V. SCRUGGS.

Voltage clamp measurements of the early transient sodium current were made with a variety of sodium concentrations on the two sides of the membrane. This current normally produces the nerve impulses. The current when there is no potential difference across the membrane is linearly proportional to the difference between the two sodium concentrations (Fick's Law) with a permeability, P, of about 10^{-4} cm/sec. The conductances, or slopes of the i–v curves for positive transmembrane potentials, when plotted in three dimensions against the concentrations on the two sides, fall on a plane with sides aNa_o and bNa_i. a is about 10^{-6} cm/(mV·sec) and b is about 3×10^{-6} cm/(mV·sec). The whole current-voltage relationship for positive potentials is just Fick's Law plus Ohm's Law.

Note that the ratio $P/(a+b)$ is about 25 mV or kT/e, as might have been expected from the Nernst-Einstein equation. This means only two constants are necessary to describe the membranes relations with sodium ions. Either a and b, the sodium acceptance from the two sides or P, the permeability and b/a, the asymmetry, are sufficient. Two different constants are required because the membrane has two different sides.

Diffraction analysis of reconstituted dogfish brain microtubules. George M. Langford.

Microtubules assembled from dogfish brain tubulin, free from accessory proteins, were negatively stained and examined by high resolution electron microscopy. The reconstituted tubules display a slight twist and a helical banding pattern which results from the superposition of the front and back sides of the tubule. The banding pattern is a moiré type pattern with a period of 96 nm. The twist most likely results from an intrinsic perturbation in the form or packing of the tubulin subunits at a period of 96 nm. This possiblity is supported by analysis of the electron microscopic images by optical diffraction and image reconstruction techniques. Optical diffraction patterns contain, in addition to the spots representing (a) the 4 nm spacing of the tubulin subunits along the protofilaments, (b) the 5 nm spacing of adjacent protofilaments and, (c) the 2.5 nm spacing derived from the groove or cleft along the center of the tubulin subunits, diffraction spots representing a 96 nm axial spacing. The 96 nm spots probably arise from a perturbation in the form or packing of subunits along the protofilaments. Images of tubules reconstructed by the optical filtering or spatial frequency filtering technique, which permits one to record obscured detail originating from one side of the tubule, show that the subunit cleft is alternately concealed and exposed along the protofilament. This observation suggests a difference in the orientation of tubulin subunits along the protofilaments. The working hypothesis is that a wave of perturbation in the orientation of subunits is present along each protofilament. The period of the wave is 96 nm and the periodically varying quantity is the degree of tilt of the subunits. The subunits alternately tilt in one direction and then the other, exposing and concealing the subunit cleft. The wave staggered in adjacent protofilaments, prodcing a 96 nm helical superlattice. This intrinsic helical superlattice in the wall of the tubule is thought to specify the binding sites for the attachment of accessory molecules.

Supported by the Steps Toward Independence Program, MBL and NIH Grant GM 24446.

Changing tides and their effect upon tissue aggregation: the neural gland of Ciona intestinalis. James W. Lash and Michael Ovadia.

Although tidal rhythms have been observed in many marine organisms, it is rare that these rhythms can be correlated with specific tissue changes. Most tidal rhythms are expressed as behavioral or activity changes. In the ascidian *Ciona intestinalis,* tidal rhythms have been found to be expressed as specific changes in the neural ganglion and the neural gland. At low tide, the neural gland is organized as a parenchyma, and is actively secreting an Alcian Blue positive substance. Wandering cells are observed in the lacunae of the gland and they contain PAS positive granules. At high tide the cells of the neural gland disaggregate and adhere to newly appearing fibers. The fibers are Alcian Blue and PAS positive. The wandering cells now have no PAS positive granules, and are scattered amidst the gland cells. During high tide, but not low tide, the neural ganglion gives evidence of active neurosecretion, with the neurosecretory granules passing out of the ganglion and down the nerve fibers. The function of the gland is unknown, and its secertory products have not yet been characterized. D. Georges (1974, *Gen. Comp. Endocrin.,* **22**: 334) has proposed that the ganglion and gland regulate spawning. The changes described above suggest that the neural ganglion at high tide secretes a substance that stimulates the neural gland to become secretory at low tide. The aggregation of the gland cells is correlated with their secretory activity. At high tide the cells adhere not to each other, as at low tide, but to newly appearing fibers.

Supported by NIH Grant HD-00380 to JWL.

Behavior of Hermissenda crassicornis (*Gastropoda: nudibranchia*) *in vertical gradients of illumination.* I. Izja Lederhendler, Edward S. Barnes, and Daniel L. Alkon.

The positive response to light was studied in 60 adults using tracking of mucous trails and direct 10-sec time-sampled observation. Animals 2 to 4 cm long were individually maintained at 15° C on a 12 light : 12 dark schedule with food always available. Individuals were observed in shallow aquaria (32×32 cm) 2 to 4 hr after lights-on and lights-off. All animals started in the center of the field. For 30 individuals, stimulation was provided by a fiber optic illuminator using a 150 W tungsten halogen quartz source positioned 30 cm above the center. The remaining animals received the same stimulation positioned 4 cm from the side. In each case, the most intense illumination [13 joules/($m^2 \cdot$sec)] was directly below the source and decreased with increasing distance to 0 joules/($m^2 \cdot$sec) 14 cm from the center. Distributions of locations at 50 sec intervals indicated: during the light phase, *Hermissenda* individuals started in the most intense region, moved to intermediate distances, and remained there. If the animals started in dim light, they moved toward the more intense areas but remained in the same intermediate zones of light intensity. During the dark phase, they moved away initially but after 15 min began moving toward the light. Preliminary measurements of linear velocity, path deviation from linearity, and orientation are systematically related to distance from the center of illumination and suggest that ortho- and klinokinesis as well as taxis are all components of the general behavioral processes. Their relative contributions are currently being investigated behaviorally and electrophysiologically.

This research was supported by the Intramural Research Program, NINCDS, NIH.

Preliminary observations on the visual pigments and behavioral ecology of selected fishes. JOSEPH S. LEVINE AND EDWARD F. MACNICHOL, JR.

Microspectrophotometric investigations of the visual pigments of selected fishes indicate that both the spectral nature of the visual environment and the behavioral ecology of the organism are important in determining the spectral locations of their pigments.

Generalized midwater species from a variety of tropical freshwater environments possess sets of visual pigments with absorption maxima in the ranges 590 to 620 and 510 to 540 nanometers (double and/or twin cones), 450 to 465 nm (single cones), and 510 to 540 nm (rods). These values approximate those of many temperate freshwater species, some of which lack the blue sensitive pigment. Both tropical and temperate marine species exhibit somewhat shorter-wave pigment sets, with twin or double cones absorbing maximally in the range 520 to 560 nm, and singles containing a blue-sensitive pigment at about 460 nm. No red-orange sensitive pigments have been found in saltwater fishes to date.

These data alone suggest that the environmental light regime is the major determinant of visual pigment sensitivity. Unfortunately, many of these fishes wander widely in nature, often covering several photic categories.

Additionally, certain surface-dwelling and shallow-water tropical freshwater species from the same environments as the "red-sensitive" group possess extreme shortwave-shifted pigment sets, with a spectral range of 410 to 570 nm. This pattern spans several phylogenetically remote families including the Osteoglossidae, Poecilidae, Cyprinodontidae and Cyprinidae. Conversely, looking within the family Cyprinidae, bottom-dwelling species such as *Labeo* exhibit pigment systems similar to those of the catfishes, while *Danio,* a surface active species, possesses a pigment set reminiscent of the Poecilidae.

Comparisons of rod and cone pigments in a number of species further reveal that one cone pigment is often matched closely to the rod pigment. Other pigments offset to longer wavelengths, or to both longer and shorter wavelengths, may be found in each species depending on their behavioral microhabitat preferences.

Supported by a Sigma Xi Grant-in-Aid and NSF Doctoral Dissertation Improvement Grant to J.S.L., and the generous support of the Rowland Foundation and other non-governmental donors to E.F.M.

Evidence for intermediate filaments in the micronuclear spindle apparatus of Paramecium bursaria. L. M. LEWIS.

Whether or not spindle elements in addition to microtubules play a role in chromosome movement is unresolved. In micronuclear spindles of *Paramecium bursaria,* several authors have reported non-microtubular fibrous elements hypothesized to be either a storage form of tubulin, or an actinomyosin system that pulls chromosomes during the early phases of chromosome movement. Most of the non-microtubular fibrous elements disappear by late anaphase as microtubules become the preponderant spindle element. Prefixation with glutaraldehyde in 0.5% lettuce tea reveals a microfilamentous meshwork in mitotic prophase. The microfilaments in the meshwork have diameters between 7 and 16 nm. Prefixation of meiotic spindles with glutaraldehyde in 0.025 M collidine buffer reveals individual filaments with a uniform diameter between 100 and 120 nm. Hence, the non-microtubular spindle elements resemble intermediate filaments observed in mammalian cells. Treatment with cytochalasin B does not affect the formation of the non-microtubular spindle elements. Postfixation with 0.1% osmium in phosphate buffer at pH 6 for 15 min at 4° C does not affect preservation of the intermediate-like filaments. During early anaphase of meiosis I, true kinetochores attach to spindle microtubules. Chromosomes were also observed during mitotic prophase in living cells using time lapse microcinematography.

Supported by a Steps-Toward-Independence Fellowship from the Marine Biological Laboratory and an Academic Research Committee Grant from Millersville State College.

Electronic conduction in proteins. T. JOHN LEWIS.

Several proteins, when complexed with methylglyoxal, exhibit enhanced electrical conductivity (see Gascoyne and Pethig, 1978, *Biol. Bull.*, this issue) and theoretical calculations based on polypeptide structure show that they can be described in terms of delocalised electron energy bands. Work is in progress to relate these experimental and theoretical features using the now well-accepted physics of the amorphous solid state. A model has been developed in which methylglyoxal and the states of the protein valence band act as acceptor-donor pairs so that the protein then behaves as a "hole" or p-type semiconductor with hole transport along the polypeptide backbone. As the experiments have indicated, long-range transport of holes and therefore of electrons across protein-protein interfaces can occur. This is difficult in the dry state but would become relatively easy in a biological environment.

Consideration has also been given to the cellular implications of such long-range hole conduction and attendant polarization. Confinement of the holes to the polypeptide backbones implies their directed transport through protein networks and the possibility of long-range redox reactions along pathways under the remote control of acceptor molecules. Long-range polarization, also under acceptor control, will have a strong ordering and aligning influence on protein molecules. Long-range electron transport and polarization mechanisms become increasingly significant as cellular processes in view of the growing evidence for an ordered protein-matrix cytoskeleton in cells.

The work is sponsored by the National Foundation for Cancer Research.

Voltage dependent conductances in Limulus *ventral photoreceptors.* JOHN LISMAN, GORDON FAIN, AND MICHAEL SWAN.

Voltage-dependent currents were measured in the dark using a two microelectrode voltage clamp technique. Net inward and outward currents were evoked by depolarizing pulses (holding potential = −70 mV) after correction for leakage currents. The inward current was reduced ($< 50\%$) by removing Ca^{++} (substituting Mg^{++}). The inward current was reduced by 10 mM Cd^{++}, Mn^{++}, or Co^{++}, and was completely blocked in 10 mM Ni^{++} calcium-free ASW. Outward currents for large depolarizing pulses had an initial transient followed by a maintained component. 1 mM 4-aminopyridine blocked the transient component but had relatively little effect on the maintained component. Intracellular injection of TEA greatly reduced the maintained component but had relatively little effect on the transient component. The transient outward component was not dependent on Ca^{++} entry, since it was not blocked in Ni^{++} calcium-free ASW. At holding voltages more positive than −40 mV the transient current was greatly reduced. In all these respects, the transient outward current resembles that found in snail neurons. The maintained current (measured at 1.6 sec) was reduced in Ni^{++} calcium-free ASW (by 70% at 0 mV). The component that was not blocked was carried by a voltage-dependent K^+ conductance. The component that was blocked might be carried by a Ca^{++}-activated K^+ conductance, however, a direct effect of Ni^{++} on the outward currents cannot be excluded. In calcium-free ASW, outward current was reduced during large depolarizing pulses, but was often increased during small depolarizing pulses. Intracellular EGTA injections greatly increased the plateau of light-induced current, but produced only a small reduction of outward currents ($< 10\%$). We conclude that the photoreceptor membrane has separate voltage dependent conductances for the inward current, the transient outward current and the maintained K^+ current. Our experiments do not rule out or confirm a Ca-activated K^+ conductance.

Double voltage clamp study in squid giant synapse. R. Llinás and M. Sugimori.

The relation between the voltage-dependent presynaptic calcium current and the postsynaptic current was studied by means of a simultaneous voltage clamp of both structures. The pre-terminal was clamped in a manner described previously by Llinás et al. (1976, *Proc. Natl. Acad. Sci.,* **73**: 2918). The postsynaptic element was clamped at the resting level (usually -70 mV) using a second clamp unit. The presynaptic current was measured either by the technique of Adrian *et al.* (1970, *J. Physiol.* **208**: 607) or at the high side of the current injection system. The latter technique was used to measure postsynaptic current. G_{Na} and G_K were blocked by extracellular tetrodotoxin (10^{-6}) and 3-aminopyridine (5 mM) and by intracellular tetraethyl ammonium, respectively. Steps of depolarization ranging from 10 to 140 mV (from a holding potential of -70 mV) were applied to the pre-fiber. The results indicated a linear relationship between the presynaptic calcium current (peak amplitude) and the postsynaptic current. More significantly, tail calcium currents following depolarization of the pre-terminal to the suppression level generated a fast postsynaptic current with a 200 μsec delay. The time course of this postsynaptic current ($\tau = 250$ μsec) was quite similar to that of the tail I_{Ca} ($\tau = 300$ sec).

These findings further illustrate the linear relation between I_{Ca} and I_{EPSP}. In addition, the rather parallel time courses for these two currents tend to exclude the possibility of activation of a secondary intracellular calcium release by the voltage-dependent I_{Ca}.

Research was supported by USPHS Grant NS14014.

Voltage-clamp study of the effects of temperature on synaptic transmission in the squid. R. Llinás, K. Walton, and M. Sugimori.

The effect of temperature (20 to 5° C) on synaptic transmission at the squid giant synapse was studied using the voltage clamp technique. Following the blocking of G_{Na} with tetrodotoxin and G_K with tetraethylammonium (intracellularly) and 3-aminopyridine (extracellularly) either the presynaptic terminal was voltage clamped using the technique of Adrian *et al.,* (1970, *J. Physiol.* **208**: 607), or both the pre- and post-synaptic terminals were voltage clamped as described by Llinás and Sugimori (this volume). Current clamp studies revealed two main effects of decreased temperature: increased latency of postsynaptic response 1 to 4 msec for 15° to 5° C ($Q_{10} = 4$), and a decreased peak amplitude with a Q_{10} close to 3.5. Presynaptic voltage clamp experiments have shown that, first, the latency of the "on" I_{Ca} is increased by a maximum of 500 μsec at 5° C; secondly, the kinetics of I_{Ca} are slowed; but thirdly, the current amplitude is only slightly affected, the Q_{10} for the peak current being 1.2 (for 12° to 5° C). Thus, slowing of I_{Ca} is responsible for only a small fraction of the increased latency, and a decrease of peak calcium current itself is not responsible for the decreased response amplitude.

In the double voltage clamp studies, postsynaptic current latency was found to increase at low temperature (from 300 μsec to 2 msec for 10° to 7° C). Further, for the same temperature range, the postsynaptic current amplitude decreased by one half, although the presynatpic current amplitude remained unchanged.

Finally, a study of the kinetics of the presynaptic tail currents has shown the time constant of these currents to have a Q_{10} of 2 to 2.5.

Thus, this study indicated that a change in the I_{Ca} itself does not play a very significant role in the increased latency or decreased amplitude of the postsynaptic response seen with decreased temperature.

Research was supported by USPHS Grant NS14014.

Activity of a proteolytic factor from the crab (Ovalipes ocellatus) *in radioligand assays for choriogonadotropin.* TAKESHI MARUO, C. A. ADEJUWON, A. R. SEGAL, S. J. SEGAL, AND S. S. KOIDE.

Reports that substances resembling choriogonadotropin (hCG) are produced by bacteria and nonplacental tissues imply a wide natural distribution of this factor. Among these publications is our earlier work, which demonstrates that extracts of the stomach or hepatopancreas of the marine crab (*Ovalipes ocellatus*) appear to contain substantial amounts of hCG-like material when tested in conventional radioimmunoassay (RIA) and radio-receptorassay (RRA), without the inclusion of protease inhibitors in the assay systems. We report now that the apparent hCG activity obtained from the crab tissue extracts is reduced drastically by the addition of trypsin inhibitors (TLCK, leupeptin, soybean inhibitor, antipain, crude ovalbumin) to the assay systems. The results suggest that the increase in radioligand activity (apparent hCG activity of the samples) is due to trypsin-like proteolytic activity. Purification of the crab hCG-like factor was undertaken to determine the relationship between the protease activity and the apparent hCG activity. The purification steps included affinity chromatography on Concanavalin-A-Sepharose (Con A-S), gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephadex A-50. The procedure resulted in a 1:6,400 purification by weight. On Con A-S the hCG-like factor eluted with the unbound fraction. Elution through Sephadex G-100 produced a parallelism in hCG-like activity as determined by RIA and RRA, and proteolytic activity as measured by a spectrophotometric method. The final chromatographic step on DEAE-Sephadex A-50 showed that the apparent hCG-like activity was eluted in three peaks by a linear gradient elution method. The fractions corresponding to the highest activity were pooled, dialyzed, lyophilized, and subjected to SDS-polyacrylamide gel electrophoresis. A major single band with a migratory rate corresponding to trypsin was observed suggesting that the molecular weight of the crab factor corresponds to trypsin (mol wt 23,300). The results indicate that the apparent hCG factor in crab tissue extracts is accounted for by a trypsin-like proteolytic enzyme which can hydrolyze the ligand and/or receptor in either RRA or RIA. The determination of hCG-like substances in tissue extracts cannot be considered complete or conclusive unless protease inhibitors are added to the RRA and RIA systems.

Supported in part by a grant from The General Service Foundation.

A temperature jump experiment for study of protein synthesis in the toadfish. RITA W. MATHEWS AND AUDREY E. V. HASCHEMEYER.

Incorporation of radioactive amino acids into toadfish liver *in vivo* declines precipitously below 7° C. A similar observation in *E. coli* has been attributed to failure of initiation of protein synthesis. Chain elongation is reported to proceed normally, leading to release of growing chains from the ribosome and dissociation of messenger RNA-ribosome complexes. To test this hypothesis, anesthetized toadfish were given an injection of ^{14}C-leucine at high dose (15 mM) into the hepatic portal vein. When revived and maintained at 20°, fish showed linear incorporation, 0.6% of dose/min, into liver protein for up to 20 min. The ratio of label in completed proteins S to total incorporation T (completed proteins + ribosome-bound chains) was 0.5 after 5 min, the time required for one round of synthesis at this temperature. Fish suddenly transferred to 3° after the 5-min labeling period at 20° showed no further incorporation of radioactivity into protein, as expected. The S/T ratio also did not change for over an hour, indicating a block on chain growth. If elongation occurs in the cold, as proposed above, S/T should rapidly approach 1 as the pre-labeled chains are completed and released from ribosomes. Instead, a slow rise in S/T occurred in fish maintained at 3° for up to 20 hr, consistent with an average chain assembly time of 6 hr. When fish were subjected to a second temperature jump back to 20°, S/T increased rapidly to approach 1 within 15 min. This indicates release of the pre-labeled chains and is consistent with normal 20° protein synthetic activity. The results in this system therefore do not support the idea of differential effects of cold on initiation and elongation in protein synthesis. Instead, a reversible and fully coordinated shut-down of the protein synthetic machinery is indicated.

Supported by NSF Grants PCM77–07164 and DPP77–20461.

Preparation of cell surface membrane from reaggregating sea urchin cells. ROBERT A. McCARTHY AND JAY C. BROWN.

Purified cell surface membrane has been isolated from cultures of reaggregating sea urchin cells by means of polystyrene latex beads. Eggs from the sea urchin, *Arbacia punctalata,* were fertilized in 0.004% Papain, grown to the 8-cell stage and dissociated by treatment with calcium-magnesium-free sea water. Upon dissociation, polystyrene beads (0.714 μ) were added and the cells were allowed to reaggregate in filtered sea water. The method takes advantage of the fact that sea urchin cells, when allowed to reaggregate on plastic dishes, become ameboid and will pinocytose polystyrene beads. To recover the pinocytotic vesicles, the cells were harvested, washed twice in homogenization buffer (0.35 M Glycine, 0.1 M Hepes pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 0.4 mM PMSF) and homogenized in a Dounce homogenizer. The homogenate was mixed 1:1 with 60% sucrose in homogenization buffer and a discontinuous gradient of 30, 25, 20, and 10% sucrose in homogenization buffer was layered over the sample. The gradient was spun at 25,000 rpm for 1.5 hr. The polystyrene beads were recovered from the 10 to 20% interface.

Transmission electron microscopy was used to verify that the polystyrene beads entered the cell and were surrounded by continuous membrane vesicles. Acrylamide gel electrophoresis showed that a reproducible population of membrane proteins are present. This method provides an alternative approach to classical cell fractionation techniques to investigate embryonic sea urchin cell surface membranes free from cytoplasmic contamination.

This work supported by PHS Grant #00265.

Color vision of honey-bees at natural low light intensities. RANDOLF MENZEL AND ROBERT ROSE.

Bees have a trichromatic color vision system with color receptors maximally sensitive at 350, 440, and 540 nm. Each ommatidium contains all three color receptors: 4 green, 2 blue, and 3 UV (2 long and 1 short) receptors. Therefore, each ommatidial field of view is trichromatically organized. Judging from the absolute and spectral sensitivity, all 8 long receptors are color receptors in the sense that they are all supplying input to a color coding system. There seems to be no specialized achromatic, dim light sensitive receptor. Such a system could further increase signal to noise ratio at low light intensities if the signals from all three receptor types in one ommatidium are pooled. Interneurones receiving excitatory input from all receptor types have been found in an earlier study. The result of such a pooling would be loss of color discrimination. We have performed behavioral experiments to find out if color discrimination is reduced or lost at low light intensities.

The experiments were performed with freely flying bees under natural light conditions. Bees were trained to a blue cardboard at a feeding place or at the hive entrance. Color discrimination was tested against three other colors (violet, bluish-green, and yellow) which had the same subjective brightness to the bee as blue. One-hundred and twenty tests with 7,818 choices have been carried out over an intensity range of 4.5 log units (1–30,000 footcandles). We found that color discrimination does not decrease, but increases at lower light intensities. This improvement in color discrimination was found in both training situations, with a white (bee white) or grey background (the grey chosen had the same brightness to the bee as the other colors), and with blue or bluish-green as the color to which the animals were retained. We, therefore, conclude that bees discriminate colors as long as they fly (twilight, 1 footcandle). The physiological basis for this improvement in color discrimination has yet to be established.

Isolation of an inhibitor of sponge cell aggregation from membranes. FRANCOISE J. MIR-LECHAIRE AND MAX M. BURGER.

Chemically dissociated cells from *Microciona prolifera* contain no detectable amounts of aggregation factor (MAF) on their surface, as established by Kuhns *et al.* with fluorescent antibodies. After fixation with glutaraldehyde, they will reaggregate in the presence of MAF in sea water. An inhibitor of aggregation, termed baseplate (BP) by Weinbaum and Burger, is released from these cells by hypotonic shock. When inhibitor is preincubated with MAF, it prevents aggregation of live and fixed cells. Hypotonically treated cells no longer aggregate in presence of MAF, but do so when preincubated with BP.

To determine if inhibitory activity is released from the cell surface, fractions enriched in membranes were prepared. Chemically dissociated cells were sonicated in calcium magnesium free sea water and the homogenate was centrifugd at 100,000 × g for 1 hr. The pellet is referred to as total membrane particulate fraction. Significant amounts of inhibitory activity were extracted from this fraction by 2 M sodium chloride, 0.5% Triton X-100, and 10 mM TrisHCl pH 7.2 only. The specific activity (SA = inhibitory units/mg protein) of this fraction was increased 37 times compared to the homogenate.

Plasma membranes were separated by the two phase polymer system of Brunette and Till. No significant contamination was detected by electron microscopy. There was a 250-fold increase in the specific activity of inhibitor extracted from this fraction.

Binding of the inhibitor to MAF was checked by using purified MAF(SA = 2,800 μ/mg protein) coupled to Sepharose beads. Membrane extracts with high inhibitory activity, as well as BP extracts, bind to MAF-beads at pH 6.1, but not to BSA-beads. In both cases inhibitory activity was eluted from MAF-beads in presence of Triton at pH 7.4, but not from BSA-beads or MAF-beads with no bound inhibitor. The SA after this step was 900 times that of the homogenate. This inhibitor is therefore a valid candidate for a receptor of MAF.

Supported by the Swiss National Science Foundation.

Pacemaker inhibiton in the hydroid Tubularia. CHARLES R. MORGAN, NORMAN B. RUSHFORTH, AND DARRELL R. STOKES.

Inhibitory interactions between the Distal Opening System (DOS) and three other established pacemaker systems have been examined. The systems studied were those producing Gonophore Pulses (GP), Proximal Tentacle Pulses (PTP) and Distal Tentacle Closing Pulses (DTCP). Previous studies have shown that DOS Pulses are inhibitory to Hydranth Pulses (HP) and Neck Pulses (NP). In our analyses of inhibitory effects, it was necessary to distinguish HP coupled and non-HP coupled pulses. Our results indicate that for each pacemaker system, coupled pulses are inhibited during the period when DOSP's are elicited by electrical stimulation. This results from DOSP inhibition of the HPs to which they are coupled; furthermore, coupled pulses occurring in bursts are inhibited to a greater degree than non-burst coupled pulses. In an assessment of inhibitory effects on uncoupled pulses, only the PTPs were inhibited independent of the HP system. With respect to control frequencies, significant inhibition and post-inhibitory rebound was evident for the PTPs, but not for the GP and DTCP systems. There are two possible mechanisms of inhibition suggested: first, the inhibition of PTPs and NPs indirectly through the HP pacemaker system and directly by the DOS, and secondly, the indirect inhibition of GPs and DTCPs through the HP system only (*i.e.,* no direct inhibition). The latter mechanism is sufficient to account for the depression of total activity observed in the GP and DTCP pacemaker systems.

Supported by NIH Grant MH-10734 to N. B. Rushforth and a grant from the McCandless Foundation, Emory University to D. R. Stokes.

The nitrogen uptake kinetics of Spartina alterniflora: *evidence for uptake inhibition in salt marshes.* JAMES T. MORRIS.

Four hydroponic cultures of *Spartina alterniflora* consisting of 96 plants each were maintained outdoors from mid-June to mid-December of 1977 for the purpose of measuring their uptake rates of nitrogen. Nutrient solutions composed of sea water diluted 50% with tap water and enriched with Fe, K, P, and N were supplied continuously to the cultures in a one-way flow. The cultures differed in the rates at which NH_4 or NO_3 were supplied. Uptake rates of NH_4 or NO_3 were measured on a weekly basis using a mass balance technique. The ambient concentrations of NH_4 or NO_3, temperature, and the resultant weight-specific uptake rates were used to fit models of uptake kinetics. No evidence was found for multiple uptake mechanisms for either nitrogen source and it was concluded that the one carrier Michaelis-Menten model will accurately predict the uptake rates of NH_4 and NO_3.

The maximum velocity of uptake (V_{max}) can be predicted from an exponential function of temperature and is greater for NH_4 than for NO_3 absorption. The half-saturation constants (K_m) for NH_4 and NO_3 uptake were estimated to be 0.057 ± 0.016 mg/l NH_4–N and 0.124 ± 0.034 mg/l NO_3–N, respectively. The low K_m's indicate that the efficiency of nitrogen uptake observed in this experiment exceeds that observed in salt marshes. This conclusion is reached because the concentration of available nitrogen in salt marshes should not be rate limiting considering the uptake kinetics measured in this experiment; yet, *Spartina* will respond to the addition of nitrogen fertilizer to the marsh. Therefore, some factor in salt marshes inhibits the uptake of nitrogen in such a way as to raise K_m and possibly to lower V_{max}. Circumstantial evidence suggests that uptake inhibition results from a lack of sufficient oxygen in the root zone or by a metabolic inhibitor linked with the availability of oxygen in marsh sediments such as H_2S. These results suggest that nitrogen is not an ultimate limiting factor in salt marshes.

This research was supported by a grant from the Rockefeller Foundation and is a contribution from the Ecosystems Center.

Fine structure of Labyrinthula *slime ways and its relation to cell motility.* NORIO NAKATSUJI, DENNIS SOLOMON AND EUGENE BELL.

Labyrinthula is a protozoa that makes colony consisting of many cells and a network of "slimeways" connecting these cells. Individual cells move gliding along the slimeways, and the slimeways also move by themselves. Other work using transmission electron microscopy (TEM) showed that the slimeways are bound by unit membrane and contain only "fibrogranular" materials.

We studied the fine structure of the slimeways by SEM, TEM of thin sections, whole mounts and negative staining. Whole mounts were prepared by picking up the colony onto the formvar film-polylysine-coated EM grids. They were fixed with glutaraldehyde and positively stained with uranyl acetate and critical point dried. Some grids were treated with 0.1% Triton X-100 briefly before fixation, then negatively stained with 1% uranyl acetate.

Whole mounts showed many filaments of about 6 nm diameter in the slimeways oriented parallel to the long axis. In the flattened region of the slimeways, networks of such filaments were observed. Some thicker filaments were also observed.

Negative staining showed filaments of the same dimension as observed in the whole mounts. It showed also numerous very thin filaments oriented mostly parallel to the long axis of the slimeways. In flattened region, these filaments were existing in all directions crossing one another. In some places filaments were aligned. The diameter of these filaments was 2 or 3 nm.

On the basis of antibody staining data, and the foregoing observation on the fine structure, we suggest that both actin and myosin may be present in the slimeways, and cause the motility.

Work supported by a grant from the M.I.T. Sea Grant Program.

Studies on urn cells of Sipunculus nudus: *Effects of serum, pH, and lectins on mucus secretion.* SANTO V. NICOSIA.

Urn cells, a mucociliary constituent of the benthic invertebrate *Sipunculus nudus,* will release mucus in response to serum. In order to investigate the mechanisms which regulate mucus secretion, these cells were cultured in presence of heat-inactivated rabbit serum (IRS), lectins, or of agents capable of affecting the intracellular pH. Mucus release occurred within 2 min of exposure to IRS, increased markedly in the ensuing 30 min, and ceased after 60 min. Continuous exposure to serum was required for sustained secretion, since IRS-induced mucus release ceased after transfer of cells in filtered sea water (FSW) alone. The hypothesis that mucus secretion depended on a contact-mediated activation of cell surface components was tested by culturing urn cells with lectins. Distinct secretory tails were formed by urn cells within 4 to 5 min of exposure to asparagus lectin (*Lotus tetragonolobus*) and, to a lesser extent, to *Ricinus communis* agglutinin (RCA_1). No significant secretory response was elicited by wheat germ and soy bean agglutinins, concanavalin A, or by horseshoe crab (*Limulus polyphemus*) hemolymph. Response to asparagus lectin was dose-related over a 30 to 1000 $\mu g/ml$ concentration range, and was inhibited by preincubation of this lectin with its specific monosaccharide, L-fucose. Lectin-initiated mucus release was also arrested by further exposure of urn cells to L-fucose. In order to establish if variations in pH were involved in mucus secretion, urn cells were cultured in FSW containing agents (NH_4OH, pH 9.0, or Na-acetate, pH 7.5) which may raise or lower the internal pH. Obvious mucus secretion was elicited within 4 to 5 min by Na-acetate, while resting secretion levels and secretory response to further serum challenge were markedly suppressed in ammonia-exposed cells. These results indicate that serum-induced mucus release in cultured urn cells is a contact-mediated phenomenon, and suggest a heretofore unreported role in mucus secretion for lectins and for a decrease in intracellular pH.

Supported by NICHHD Grant HD 06274-06.

Kinetics of HCO_3^-/Cl^- exchange in fish erythrocyte suspensions. Ana Lia Obaid, Anne McElroy Critz, and Edward D. Crandall.

Using a stopped-flow rapid reaction apparatus, changes in the extracellular pH of fish red blood cell suspensions were monitored under conditions in which dpH/dt was determined by the rate of HCO_3^-/Cl^- exchanges across the cell membrane. Blood was drawn by venipuncture from the Elasmobranch, *Mustelus canis* (dogfish), and by cardiac puncture from the Teleost, *Lophius americanus* (goosefish). The red cells were separated by centrifugation and resuspended to 20% hematocrit in either their own plasma (A1) or in Ringer's solution (A2). Bovine carbonic anhydrase and sodium bicarbonate were added to the cell suspensions. Experiments were performed at 25° C by mixing equal volumes of A1 with plasma or A2 with phosphate-buffered Ringer's solution in the rapid reaction apparatus while recording the time course of pH equilibration in the mixture. Experiments performed in the absence of cells confirmed that the bovine carbonic anhydrase remained active in both the fish plasma and Ringer's solutions. The rate of HCO_3^-/Cl^- exchange in dogfish red cell suspensions was 1.10 nmole/($cm^2 \cdot sec$) in plasma and 1.24 nmole/($cm^2 \cdot sec$) in Ringer's solution. These values are not significantly different from each other, and are similar to those reported for human red cell suspensions. The exchange was markedly inhibited in the presence of SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid), a potent anion transport inhibitor. Although goosefish cells exhibited some anomalous behavior, exchange fluxes for these cells were 1.79 nmole/($cm^2 \cdot sec$) in plasma, and 2.59 nmole/($cm^2 \cdot sec$) in Ringer's solution. These results suggest that the rate of HCO_3^-/Cl^- exchange in fish red blood cell suspensions is not altered by plasma factors, and that intra-erythrocytic carbonic anhydrase may be accessible to plasma HCO_3^- *in vivo*.

Tail thrust and body drag measured in swimming bluefish (Pomotamus saltatrix) *during laminar and induced turbulent flow conditions.* Christopher S. Ogilvy and Arthur B. DuBois.

Thrust and drag of swimming fish have previously been difficult to measure. In the present study two methods were used: the pressures on both sides of the tail of bluefish swimming in a 30 cm diameter water tunnel were measured by sewing pressure transducers on each side of the tail. These pressures were resolved into their forward and lateral vectorial components of force and power. The force (mass × acceleration) in the forward direction was also measured by a forward accelerometer. The peak to peak force as measured by the accelerometer (2.05 newtons at a speed of 0.48 m/sec) compared well with that calculated from the pressure measurements on the tail (1.97 newtons at 0.48 m/sec). The mean lateral power of the tail at this speed was 1.28 (newton·meters)/sec and the propulsive efficiency of the tail, calculated as the ratio of forward power to forward plus lateral power, was 0.20 s.d. ± 0.04 s.e. 0.01 and was not related to speed. The effect of turbulence on the swimming fish was investigated utilizing the accelerometer to measure body drag ($F_D = \text{body mass} \times \text{peak to peak acceleration} \div 2\sqrt{2}$). Turbulence was induced in two ways: two plates 30 cm long and 1.9 cm wide, could be lowered in front of the fish, or a bent rod could be rotated in the water using a portable electric mixer. The relationship of drag force of the swimming fish to water speed in turbulent flow remained exactly the same as in laminar flow. The maximum swimming speed of any individual fish was not altered by the induction of turbulence in the water. Tests made by other investigators on rigid streamlined models have shown that the drag force increases in turbulent conditions. Our measurements suggest that swimming bluefish do not behave like rigid models, possibly because active swimming creates positive pressures on the convex side of the flexing body, preventing boundary layer separation.

Transformation of fast and slow skeletal muscle during growth of juvenile lobsters. Mark M. Ogonowski and Fred Lang.

The development of lobster claw closer muscles was investigated from the symmetrical state in early juveniles to the asymmetrical state of adults. The adult crusher claw closer muscle is composed entirely of slow fibers, while the cutter closer muscle has about 65% fast and 35% slow fibers. However, in early juvenile stages both claw closer muscles have 30 to 40% fast and 60 to 70% slow muscle fibers. In addition, claw type is not fixed until the 5th stage.

When stained for myosin ATPase activity, a band of darkly staining fast fibers, sandwiched between lightly staining slow fibers, is found in both closer muscles of a 4th stage animal. In the late 5th stage, when claw type is fixed, one claw has greatly expanded its fast fiber population into the dorsal region of the closer muscle, while the muscle fibers in the other claw have not changed significantly. With each successive molt, the presumptive cutter claw continues to expand its fast fiber population until, by the 10th stage, the entire dorsal region is occupied by fast fibers. The other claw only slowly loses its fast fiber population and ultimately becomes the crusher.

Since the fast fiber population of one claw is expanding while the other is contracting, we looked for evidence of fiber transformation or fiber degeneration. In some cutter claws, intermediate fibers were observed in the dorsal region, between the bands of fast and slow muscle. These appeared to be transforming from slow to fast on the basis of two criteria: first, they stained as distinct fibers with an intensity intermediate between the adjacent fast and slow fibers; and secondly, they had diameters which were intermediate between the adjacent fast and slow fibers. No evidence was obtained for degeneration of slow fibers or *de novo* myogenesis of fast fibers.

Supported by grants from NSF, NIH and Boston University Graduate School.

Early course of exocytosis captured by rapid-freezing Limulus *amebocytes.* R. L. Ornberg and T. S. Reese.

Clotting proteins contained in membrane-limited granules are secreted from *Limulus* amebocytes in response to the external application of bacterial endotoxin. To determine the early morphological events leading to secretion, amebocytes frozen after inhibition in propanolol were compared with ones stimulated 5 to 60 sec after exposure to endotoxin. Several changes in the plasmalemmas of stimulated, but not inhibited, cells were apparent at the earliest times with the freeze-fracture technique: small caveolae 70 nm in diameter; oval bulges 0.25 μm in diameter; and shallow depressions with flat or convex bottoms 0.25 μm in diameter. Thin-section views of parallel treated cells prepared by freeze-substitution showed that the caveolae are not associated with secretory granules or other cytoplasmic granules; their function is unknown. The outward bulges are appositions with cisterns of endoplasmic reticulum, which appear to have swollen as a result of stimulation. The inward depressions proved to be sites of initial contact of the plasmalemma with secretory granules. The next step in secretion, seen more frequently at 15 sec, was the appearance of a small neck connecting the inside of a granule with the surface. After 15 sec these necks gradually widened and deeper granules formed similar openings in the membranes of previously released granules. We conclude that exocytosis in amebocytes begins at a contact between the plasmalemma and granule membrane which, in distinction to similar contacts reported at other secretory cells, has a full complement of intramembrane particles. The membrane rearrangement leading to the initiation of exocytosis is not coextensive with this contact but, instead, is very small.

Comparison of ciliary and flagellar membrane composition. TIMOTHY OTTER.

The protein composition of ciliary and flagellar membranes has been examined by comparative SDS gel electrophoresis. Body cilia from *Paramecium caudatum* and sperm tail flagella from *Arbacia punctulata* were treated with the nonionic detergent Nonidet P-40 to solubilize the membrane selectively and leave the axoneme intact. The protein composition of both the solubilized or "membrane" fraction and the axonemal fraction were analyzed by 5% SDS-polyacrylamide gel electrophoresis. Both Tris-glycine and Tris-acetate buffered systems were used The membrane of *Paramecium* cilia contains two major species on Tris-acetate gels: a broad band containing 40% of the total membrane protein migrating with an apparent molecular weight of 235,000 daltons, and a second species migrating at 55,000 daltons. This lower molecular weight band was resolved on Tris-glycine gels into two equimolar bands which comigrate with authentic axonemal α and β tubulins. No glycoproteins were present as determined by periodic acid-Schiff staining. The membrane from *Arbacia* sperm tails contains four major bands on Tris-acetate gels: a closely spaced doublet at 150,000 daltons, and bands at 78,000 daltons and 55,000 daltons. Separation on Tris-glycine systems produces a different pattern; a band at 78,000 daltons is still seen, but the 150,000 dalton component runs as a single band and the 55,000 dalton species splits into two bands which comigrate with the α and β subunits of authentic axonemal tubulin. Finally, a PAS-positive band was observed coincident with the α subunit of tubulin on Tris-glycine gels. These results suggest that there is a glycosylated tubulin in the membrane of *Arbacia* sperm tails.

This work was supported by USPHS Grant GM00265 to the Physiology Course.

Fine structure of synaptosomes and cells rendered membrane-free by detergent extraction. HARVEY B. POLLARD AND GEORGE D. PAPPAS.

The internal structure of cells may be based on a contractile fibrous reticulum or network and we were interested in whether cellular membranes and organelles such as those found in nerve terminals interacted with the network in any specific manner. Our approach to this question was to fix synaptosomes with glutaraldehyde and then extract membranes and other constituents with detergents such as Triton X-100 and sodium dodecyl sulfate (SDS). This treatment of fixed synaptosomes resulted in differential loss of plasma and organelle membranes, depending on the detergent, though in most cases the ensemble of organelles (mitochondria and synaptic vesicles) remained intact. Extraction in Triton X-100 resulted in complete or partial loss of plasma membranes with junctional complexes being retained and, in some cases, synaptic vesicle membranes were partially dissolved away, leaving only intact cores. Vesicles also appeared to be associated with fibrous elements, both in control and detergent-treated cases. Extraction with SDS resulted in complete loss of synaptosomal membranes and chemical analysis revealed that SDS treatment of fixed synaptosomes extracted 82 ± 5% of the total lipid-soluble material while extracting less than 10% of the total synaptosomal protein. Osmium tetroxide post fixation was omitted from some samples of synaptosomes in order to avoid protein extraction, and the result was a dramatic enhancement of fibrillar cytoplasmic elements in the spaces between organelles. We also obtained similar morphological results in studies on human red cells and blood platelets. We interpret these results to indicate that organization of organelles in nerve terminals and cells is closely related to the integrity of the fibrous cytoplasmic reticulum, but independent of the lipid phase of membranes. However, whether organelles are held in cytoplasm by simple trapping or by specific interaction, or a combination of these factors, remains to be resolved.

An ecological model of anaerobic decomposition processes: validation. JAMES P. REED.

This model is designed to simulate the disappearance of material at different depths in sediment. It predicts rates of decomposition from a Monod-type of equation based on substrate concentrations and microbial biomass. The concentrations of end products produced from decomposition are a function of the Gibbs free energy of reaction, the cell maintenance requirements and population turnover time. For the sand-detritus system used to validate the model, there are two possible processes which provide substrate for the sulfate reducing microbes.

One process is autolysis and leaching of the detritus. The other process is the release of material by the cellulose fermenters.

Model results agree well with experimental results using autolysis and leaching as the sole substrate source. The cellulose fermenters do not appear to serve as an important source of substrate for the sulfate reducers during the first six months of decomposition. Sensitivity analyses indicate that only the rate of supply of substrate is critical. Varying the quality of substrate from a high energy carbohydrate to a low energy fatty acid produced only a 20 to 30% change in the rate of sulfate reduction. A two to ten-fold change in any of the remaining parameters accounts for less than an eight percent change in the sulfate reduction rate.

Cation specificity for aggregation and for aggregation factor stability of the marine sponge, Microciona prolifera. Doug Rice and Tom Humphreys.

Cation activity of aggregation factor (AF) was assayed by the serial dilution method of Humphreys. When compared to 0.020 M Ca^{++}, 0.010 to 0.075 M Mn^{++} had 25% activity, 0.050 to 0.100 M Cd^{++} had 50% activity, 0.005 M La^{+++} and 0.020 M Nd^{+++} had 50% activity, and 0.020 M Eu^{++} had 25 to 50% activity. Cations that lack activity at 0.020 M and 0.100 M include: Mg^{++}, Sr^{++}, Ba^{++}, Cr^{++}, Cr^{+++}, Pd^{++}, Co^{++}, Cu^{++}, Ni^{++}, Fe^{++}, Eu^{+++}, Gd^{+++}, Tl^{+++}, Tb^{+++}, UO_2^{++}, VO^{++}, and putrescine.

Stability was tested by dializing 0.200 ml of 640 μ/ml AF in 0.001 M Ca^{++} against 0.100 liter of a 0.001 M or 0.020 M conc. of the following cations in MBL – SW – 1: Mg^{++}, Sr^{++}, Ba^{++}, Mn^{++}, La^{+++}, putrescine, and 0.075 M Cd^{++}. Then activity was assayed as above in Ca^{++}. 0.001 M La^{+++} stabilized 25 to 50% of AF activity, while a 0.020 M solution stabilized 50%. 0.020 M Sr^{++} and Ba^{++} stabilized 100%, while 0.001 M failed. 0.075 M Cd^{++} stabilized 100%, while 0.020 M gave 50% activity and 0.001 M failed to stabilize AF. Mg^{++}, Mn^{++}, and putrescine did not stabilize AF.

Interaction of Salmonella typhimurium *lipopolysaccharide (LPS) with the amebocytes of* Limulus polyphemus. Frederick R. Rickles, Paul D. Rick, Peter B. Armstrong and Jack Levin.

In *Limulus* an important defense mechanism against invading pathogens involves the release of a clottable protein from the blood cells (amebocytes), triggered by bacterial endotoxin (lipopolysaccharide—LPS). In this study we have examined the conditions whereby LPS purified from a conditional mutant of *S. typhimurium* (G30) and labelled with 3H by the Wiltzbach procedure will associate with the amebocyte cell membrane. At 4° C, 3554 ± 259 (1 s.e.m.) dpm (16.9% of added 3H-G30 LPS) bound to 0.5×10^6 amebocytes after one hour of incubation. Preincubation of the cells with unlabelled heterologous LPS at 100-fold excess concentration reduced the cell-associated LPS to 1905 ± 45 dpm (9.2% of added 3H-G30 LPS), while pulse-chase experiments with autologous LPS (unlabeled) reduced cell-associated 3H-G30 LPS to 1972 ± 182 dpm (9.8% of added material). Preincubation of the 3H-G30 LPS with 100-fold excess of polymyxin B (PB), a cyclic peptide antibiotic which binds to the lipid A region of the molecule, reduced binding to 1257 ± 126 dpm (6.2% of added 3H-G30 LPS). Kinetic experiments at room temperature and 4° C revealed a cyclic pattern of loading and unloading of 3H-G30 similar to that seen with mammalian cells, which may represent shedding of LPS-receptor complexes. Since PB can inhibit other cellular functions of LPS (mitogenicity, complement activation, tissue factor activation) at 10-fold less concentration, we would suggest that interaction of LPS with amebocyte membranes may occur at sites other than the lipid A portion of the molecule. Using 5-fold increases in concentration of 3H-G30, binding was reduced progressively from 11 to 4%, suggesting saturation of putative binding sites. In summary, we have established this system as a model for LPS interaction with cell membranes, in general, and future experiments with *Limulus* amebocytes should provide more information regarding this complex cellular defense system.

Supported by the Medical Research Service of the Veterans Administration and by Grant #1R01 Ca 22202-01A1 awarded by the National Cancer Institute, Department of Health, Education and Welfare.

Possible association of tubulin with coated vesicles. MARGARET S. ROBINSON.

A 55,000 dalton protein has recently been found in preparations of coated vesicles isolated from dissimilar tissues (Woods *et al.*, 1978, *J. Cell. Sci.*, **30**: 87). This protein was further characterized, since it has the same molecular weight as tubulin.

Coated vesicles were purified from calf brain using three sucrose density gradient centrifugation steps. The sucrose concentration was reduced after each step by dialysis to minimize osmotic shock. After the final purification step, nearly all the contaminating material which could be seen by negative staining had been removed.

A 55,000 dalton protein which comigrated with calf brain tubulin was seen in purified preparations assayed by SDS polyacrylamide gel electrophoresis using Tris-acetate buffer. When run on Tris-glycine gels, this protein split into two bands which comigrated with the α and β subunits of tubulin.

During the purification procedure, the 55,000 dalton protein copurified with clathrin, a protein known to be associated with coated vesicles, suggesting that the 55,000 dalton protein is not a contaminant from extraneous tubulin. The protein does not appear to be PAS-positive, and it is only minimally extractable by 1% Triton X-100. However, the possibility that it might be associated with the coated vesicle membrane has not been excluded.

Anderson and coworkers have shown that specific membrane proteins may be clustered at the coated regions of the plasma membrane before being internalized into coated vesicles (1975, *Proc. Nat. Acad. Sci. U.S.A.*, **73**: 24). Since tubulin has been implicated in anchorage modulation of the cell surface (Edelman, 1976, *Science*, **192**: 218), it is interesting to speculate that tubulin might be involved in preventing the lateral diffusion of these clustered proteins.

This work was done during the Physiology Course, and was supported by NIH Grant GM-00265.

Effect of phototherapy on the developing sea urchin. PNINA G. ROSENKRANZ, AMY J. BEHRMAN, DORIA R. GORDON, HERBERT S. ROSENKRANZ, AND WILLIAM T. SPECK.

Phototherapy (PT), *i.e.*, illumination with white or blue lights, is utilized routinely in the treatment of neonatal jaundice. Earlier work in our laboratories had indicated that PT was mutagenic and possessed DNA-modifying properties for prokaryotic and eukaryotic cells. These findings suggested that PT may preseent a health hazard to newborn infants. In view of the ability of visible light to penetrate gonadal tissues of the newborn and the possibility of light-induced genetic and developmental abnormalities, we investigated the effects of PT on the gametes and developing embryo of *Arbacia punctulata*.

Illumination of sperm with white light resulted at low doses in dose-dependent developmental abnormalities. At higher doses the ability to fertilize normal eggs was lost. Illumination of unfertilized eggs led to (a) the accumulation of abnormal plutei at low doses, (b) developmental arrest prior to blastulation at intermediate doses, and (c) loss of fertilizability at high doses. Illumination of developing embryos resulted in developmental abnormalities. The amount of light used was only a fraction of that received during a 24-hr period by a baby undergoing PT. With the use of appropriate filters, it was shown that maximal effects were seen at or near 450 nm. Similar embryotoxic effects were seen when gametes were "pulsed" with light in the presence of riboflavin.

These findings suggest that biologic effects are due to the photoactivation of an intracellular chromophore, possibly riboflavin (RBF, $\lambda_{max} = 450$ nm). RBF is known to photogenerate singlet oxygen which is capable of modifying cellular DNA.

The results suggest that because newborn infants are relatively transparent to light, PT may have an effect on their gonadal and differentiating tissues.

Optical signals from squid giant axons following perfusion or superfusion with potentiometric probes. B. M. SALZBERG.

Changes in extrinsic absorption, fluorescence, or birefringence in response to voltage clamp steps were measured from squid axons following the addition of various dyes either by superfusion or by internal perfusion. The behavior of potentiometric probes, applied to excitable membranes from different sides is of interest because such molecules are presumed to detect electrical activity of intracellular membrane systems, and might also be used, following microinjection in the manner of Lucifer Yellow, to indicate electrical events in the membranes of fine axonal or dendritic processes.

No potential dependent fluorescence signal was detected following perfusion with Lucifer Yellow CH (0.1%) itself. However, the presence of this intracellular marker did not block the potential dependent absorption signal exhibited by a Merocyanine-rhodanine dye (WW 375) added by internal perfusion. As in the absence of Lucifer, and consistent with its fixed negative charge rendering WW 375 impermeant, the optical signal was opposite in sign, though similar in size to that obtained following addition extracellularly.

A different result was obtained following perfusion with Nile Blue A, a cationic dye exhibiting potential dependent changes in fluorescence when applied to squid axons from the outside. Axolemma stained with Nile Blue A exhibits an increase in fluorescence upon depolarization, whether it is stained from the outside or by internal perfusion, as though the dye were membrane permeant.

It has been reported (Valenzeno and Pooler, 1978, *Biophys. J.*, **21**, 43a) that 2,4,6-trinitrobenzene sulfonic acid (TNBS) blocks the photodynamic damage produced by the potentiometric probe Merocyanine 540. We found that 5 mM TNBS (pH 7.3) blocks 90% of the M 540 phototoxicity, but also reduces the size of the optical signal by a factor of 4 or 5. A concentration of this agent which optimizes the ratio of protection: signal could be determined, but we have not done so.

Supported in part by National Science Foundation Grant BNS-7705025 and a Steps Toward Independence Fellowship from the Marine Biological Laboratory.

Feeding mechanisms in the ctenophore Pleurobrachia pileus. RICHARD A. SATTERLIE.

Consumption of zooplanktonic prey by the cydippid ctenophore *Pleurobrachia pileus* occurs in three steps: prey capture by the tentacles, prey transfer from the tentacles to the mouth, and ingestion. Prey capture occurs by chance contact of the prey with the adhesive colloblast cells which cover the tentacle surface. A series of brief tentacle contractions occur in response to struggling of the prey. The prey transfer sequence involves spinning of the body in the plane of the tentacles, always initiated toward the prey-bearing tentacle. As a result of several rotations, the tentacles are wrapped around the body, usually in contact with the lips. The portion of the tentacle in contact with the mouth is actively surrounded by the lips and pulled through by tentacular twitches. Ingestion occurs when the part of the tentacle containing the prey is brought through the lips.

Extracellular electrophysiological recordings from the body surface of *Pleurobrachia* reveal two distinct conduction systems, possibly nerve nets, which modify the beating of the ciliary comb plates, the effectors of locomotion and spinning. Activity in one conduction system inhibits the beat of the plates, while that of the other induces an increase in beat frequency. The latter is agonized by eserine and blocked by curare, neither of which modify the inhibiting system. Tentacle stimulation produces activity in both conduction systems, which, in turn, produce the initial spinning movements through their interaction with the comb plates. Centrifugal forces acting on the aboral statocyst presumably reinforce and prolong the spinning movements.

This investigation was supported by a Grass Foundation Summer Fellowship.

Density dependent mechanisms in sperm whale populations. DAVID S. SCHIMEL, WILLIAM S. LITTLE, JR., AND DANIEL B. BOTKIN.

A model of population dynamics and social behavior was developed in order to examine the effects of changing sex ratio and density that occur with harvesting. Sperm whales are divided into four social groups: pods made up of females and young; harem masters, adult males associated with specific pods; adjacent males, who remain on the breeding ground during the breeding season, and who may become harem masters; and migrating males who leave the breeding ground during the breeding season. The model includes searching by adjacent males for pods unaccompanied by harem masters. This occurs throughout the breeding season. The males move randomly, unless they come within detection range of a pod, in which case they tend to move towards it.

Results show that a decrease in the sex ratio (males/females), as would occur with selective harvesting will produce a decline in fecundity. This effect is largest when the sex ratio is below 0.13. The relationship shows a sigmoidal form.

As female density on the breeding ground decreases, fecundity increases. This effect is most pronounced when the population is reduced to 50% or less of its original density. If density is increased while holding the sex ratio constant, an increase in fecundity is observed.

In summary, time spent searching by males for females can affect the population dynamics of sperm whales, and that these effects will be largest in heavily exploited populations.

This research was supported by the Whale Protection Fund.

A freeze-fracture study of the skate electroreceptor. T. J. SEJNOWSKI AND M. L. YODLOWSKI.

The sensory epithelium lining the ampulla of Lorenzini in the skate (*Raja*) was examined with the freeze-fracture technique. At its apical surface, the tops of the sensory cells protrude from the surrounding supporting cells; extensive and complete tiers of tight junctions encircle the apex of each sensory cell. This anastomotic system may be responsible for the electrical isolation observed between the inside and outside of the sensory epithelium. There is a striking difference in particle size and density on the P face of the sensory cell across the tight junction complex. The apical surface has a low density of large particles which may represent the Ca^{++} channels believed to mediate sensory transduction. The lateral surface has a very high density of small particles.

Each receptor cell makes several ribbon synapses on a sensory axon applied to its basal surface. The ribbon is a flat plate of fuzzy material covered with synaptic vesicles extending from near the tip of the synapse, which invaginates the axon, to the upper arch of the synapse, where its membrane is reflected around supporting cell processes. Near the tip, the vesicles stop abruptly at a narrow constriction, where there is a striking row of large particles on the E face of the postsynaptic axon—particle aggregates of this type often represent receptors, so the constricted region may be the active zone of this synapse. In the vesicle-free zone around the tip there are large particles on the P face of the presynaptic sensory cell and the P face of the axon. The large particles in the two membranes are coextensive and arranged in a crystalline array, indicating an intermembrane junction rather than an active zone. At the top of the synaptic arch, dimples are commonly seen on the P face of the receptor cell. Since they are seen in the same region where coated vesicles are found in thin section, these dimples may be sites of endocytosis.

Differential holography of squid giant axons during excitation and rest. M. SHARNOFF, N. J. RUMER, L. B. COHEN, B. M. SALZBERG, M. B. BOYLE, AND S. LESHER.

Differential holograms recorded with light scattered by segmental ganglia have been reported to yield reconstructed images which register individual action potentials in the somatosensory neurons of the leech (Sharnoff, Henry, and Bellazza, 1978, *Biophysical J.*, **21**: 109a). In an effort to understand the origins of this effect, we have made holograms of space-clamped giant axons from the squid, of *Loligo pealei,* using analogous protocols. In all of the experiments two holograms were superimposed on the emulsion, using 514.7 or 632.7 nm laser light, scattered at 10° to 20° from the incident direction; the scattering plane being perpendicular to the axon, and also to the electric vector of the incident wave. The hologram was differential or not, depending upon whether or not a 180° phase shift was introduced in the reference beam between shutter openings. In several experiments, the mean brightness of the images derived from the differential holograms averaged less than 0.1% of those obtained from holograms in which the phase was not shifted.

Two experimental paradigms were employed. In one protocol two exposures were made, one with the axon at rest and the other during membrane action potential or a voltage-clamp step (50 to 150 mV, hyperpolarizing or depolarizing). In the second protocol, the axon was stained with merocyanine 540, a dye which exhibits potential-dependent changes in absorbance when bound to membrane. In this case the first holographic exposure coincided with a 150 mV hyperpolarizing and the second with 150 mV depolarizing step. For both protocols, control differential holograms were made in which the axon remained at rest during both exposures. No consistent differences could be discerned between results obtained from stimulated axons and those obtained from controls.

Supported by NIH Grant NS 08437 and NSF Grant BNS 7705025, and a Steps Toward Independence Fellowship to BMS.

Studies of H2A-H2B depleted chromatin. Roberta Shahin, Jamie Grifo, and K. E. Van Holde.

In an attempt to determine which of the four histones of the nucleosome are required to maintain DNA in its supercoiled configuration about the core particle, we have analyzed H2A-H2B depleted nucleosomes.

Chromatin is isolated from chicken erythrocytes by lysis in low ionic strength buffer, followed by an extraction of H1 and H5 in 0.65 M NaCl, 5 mM Tris, 0.35 mM EDTA, and 0.1 mM PMSF, pH 7.2. Individual nucleosomes are obtained by subjecting the H1–H5 depleted preparation to digestion by micrococcal nuclease at 37° C for 30 min. The digested material is spun through a 5 to 20% continuous sucrose gradient, and a monomer nucleosome consisting of a 140 base pair length of DNA and two each of histones H2A, H2B, H3, and H4 is recovered. H2A and H2B are removed from the nucleosome by extensive dialysis in 1.2 M NaCl at 4° C and the depleted monomer separated out from free histone by column chromatography on Sephadex G-100.

The depleted particle ran as a single band, distinct from either free DNA or non-depleted nucleosomes on a 6% nondenaturing acrylamide gel, visualized by ethidium bromide staining. Our preparation was found to contain at least 20% H2A-H2B depleted particles, the only other material present being free DNA, and had a sedimentation value of approximately 6S. Free DNA sediments at 4 to 5S, and intact nucleosomes at 11S. A preparation in which 20% of the sample sediments at 11S would show a step at 11S in the sedimentation profile; none is seen in our experiment. This indicates that an H3–H4 tetramer is not sufficient to form a compact nucleosomal particle.

This preliminary result is indication of an unfolding of the H2A-H2B depleted core particle, a result consistent with reconstitution experiments in which 140 base pair DNA reconstituted with two each of H3 and H4 is found to sediment at 6.45S (D. Crothers, personal communication). Since the H2A-H2B depleted nucleosome is cleanly resolvable from free DNA by acrylamide gel electrophoresis, a purified fraction of H2A-H2B depleted core particles should be obtainable through preparative electrophoresis, thus allowing further analysis.

This work was supported in part by PHS Grant GM-00265.

Scanning electron microscopy of Labyrinthula sp. *cells and colonies grown on glass cover slips and* Zostera marina. D. Solomon, S. Sher, N. Nakatsuji, and E. Bell.

Vegetative cells and colonies of *Labyrinthula* sp., a colonial eukaryotic protist, were examined by means of SEM after inoculation on substrates, which included uncoated and poly-d-lysine coated glass cover slips and viable fronds of *Zostera marina* which had been wiped clean of other organisms. Intact colonies and vegetative cells separated from the "slimeway" network by gentle douncing were incubated on the substrates for periods ranging from 10 min to 36 hr. Specimens were prepared for SEM by fixation in 2% glutaraldehyde in diluted sea water, post-fixation in OsO_4, dehydration in increasing concentrations of ethanol and critical point drying. SEM supported and extended observations made with phase-contrast, nomarski, and polarization optics that the "slimeway" network is composed of filopodial-like processes which anastomose frequently. Some foci of fusion were identified as disc-shaped hubs, 1 to 2 microns in diameter, with 5 to 12 radial filopodial-like processes. Standard error of the mean also supported our previously-reported finding that the "slimeway" network possesses lamellipodial-like processes up to 200 sq. microns in area. Regular arrays of up to 60 spherical bodies, 0.5 to 1 micron in diameter, were observed in the cortex of the vegetative cells. These bodies may correspond to electron-dense spheres reported by others in TEMs after post fixation in OsO_4, and to the optically refractive bodies on which we have reported previously. Two distinct rails, 0.1 micron in diameter, were occasionally observed extending from one end of the vegetative cells. The surfaces of cells appeared to be traversed by variable numbers of cables which extended onto the substrate in various directions. We suggest that these cables may represent developing "slimeways" along which cells may travel.

This work was supported in part by grant to D.S. from the M.I.T.-U.R.O.P. and by a grant from the M.I.T. Sea Grant Program.

Long latency bursting in the hydroid Clava. Darrell R. Stokes, Norman B. Rushforth, and Nancy M. Rushforth.

Electrical stimulation of the behaviorally quiescent colonial hydroid *Clava squamata* gives rise to a program of Delayed Burst Pulses (DBPs) with unusually long latency (ca. 5–30 sec). Programs of up to 66 bursts have been elicited by a single shock, but usually a preparation requires multiple shocks to initiate programs consisting of 1 to 6 bursts (of 5–15 pulses) in a 2 min period. Extracellular recordings with suction electrodes from intact polyps and body parts indicate a localized region of burst initiation in the distal hydranth (tentacles, hypostome and subhypostome). Pulses within a burst show facilitation and are associated with contraction of the body column and tentacle depression. DBPs may initiate Contraction Pulses (CPs) in interconnected polyps but are distinguished from them by: first, higher threshold; secondly, higher conduction velocity; and thirdly, greater magnesium sensitivity. Photic stimulation induces both DBPs and CPs, whereas the amino acids lysine, alanine, glutamine, histidine, glycine, asparagine and tyrosine at 10^{-4} M activate only CPs. The latency to the first pulse in the DBP program, the number of pulses/burst, and the interburst interval are relatively refractory to changing regimes of electrical stimulation, *i.e.*, intensity, number of shocks, and the temporal sequence of stimulation. Thus, a hydroid with a relatively simple nervous system can produce programs of bursts with unusually long latencies, which are resistant to modification by electrical input.

Supported in part by NIH Grant MH-10734 to N. B. Rushforth and a grant from the McCandless Foundation, Emory University, to D. R. Stokes.

Metacercariae of digenetic trematodes from ctenophores and medusae in the Woods Hole, Massachusetts, area. Horace W. Stunkard.

It has long been known that certain hydrozoan and scyphozoan medusae and ctenophores in the Woods Hole, Massachusetts, area, harbor unencysted metacercariae of digenetic trematodes. In an abstract, Stunkard (1967, *Biol. Bull.*, **133**: 488) reported natural infections in *Bougainvillia carolinensis, Gonionemus vertens,* and *Chrysaora quinquecirrha.* He also reported an unidentified ocellate, trichocercous cercariae from *Anachis avara,* whose morphology closely paralleled that of certain metacercariae in the jelly-fishes. The present report is concerned with five species of trematode metacercariae taken from medusae and ctenophores. Three species have been identified and the life cycle of two have been published: first, that of *Neopechona pyriformis* (Linton, 1900) (Stunkard, 1969, *Biol. Bull.*, **136**: 96–113) and secondly, *Lepocreadium setiferoides* (Miller and Northrup, 1926) (Martin, 1938, *Biol. Bull.*, **142**: 326–334). The life-cycle and developmental stages of the third species, *Lintonium vibex* (Linton, 1900) (Stunkard and Nigerelli, 1930) has now been published (Stunkard, 1978, *Biol. Bull.*, **155**: 383–394). Two additional metacercariae have been identified with ocellate, trichocercous cercariae: one from *Mitrella lunata,* the other from *Nassarius trivittatus.* The morphology of the cercaria from *M. lunata* has been worked out; it is probably a species of *Opechona,* but the definitive host is yet unknown. The other species under investigation, from *N. trivittatus,* is large, with enormous ocelli and 27 pairs of setae on the tail. The body is so thick and opaque that morphological details are not yet determined. Figures of stages in the life-cycles of these trematodes will be presented.

Investigation supported by NSF DEB-74-14534-AO1.

EGTA itself reduces outward current in Limulus *ventral photoreceptors.* MICHAEL SWAN, GORDON FAIN, AND JOHN LISMAN.

The effect of externally applied Ca-EGTA on currents (corrected for leakage) evoked by depolarizing voltage steps was studied using a two-microelectrode, voltage clamp technique. The maintained currents were measured at 1.6 seconds and the holding potential was −70 mV. The Ca-EGTA solution consisted of ASW + 1.17 mM $CaCl_2$ + 1.17 mM EGTA (Eastman and Sigma), to give a free Ca concentration equal to that of ASW. All solutions had a pH of 7.8. EGTA reduced the maintained outward current (at V = +30 mV the average reduction was 29%), but had little effect on either the inward or transient outward currents. Recovery was slow and rarely complete (varying from 75 to 110% of control). EDTA gave the same results. To determine whether EGTA action was due to a leakage giving rise to intracellular EGTA, we injected EGTA directly into the cytoplasm. EGTA injection (calculated upper limit = 30 mM) resulted in a change in the waveform of the light-induced current shown by Lisman and Brown to be characteristic of a large intracellular concentration of EGTA, but the dark maintained outward current was reduced by less than 10%. The effect of Ca-EGTA was still observed after blocking the inward current with 10 mM Ni^{++} calcium-free ASW. Since Ni^{++} calcium-free ASW should block a Ca^{++} activated K^+ current, EGTA's major effect appears to be on the voltage dependent outward current. Pepose and Lisman have shown that this maintained outward current is carried by K^+. In conclusion, EGTA itself reduces the voltage-dependent K^+ conductance by acting on the outside of the cell. In addition, these experiments, as well as Kostyuk's, show the need to do controls when using EGTA, especially when studying a Ca^{++}-dependent K^+ conductance, since the depression in outward current observed by the lowering of Ca^{++} with EGTA might in part be simply due to the direct effect of EGTA itself.

Charge transfer and dielectric constant. ALBERT SZENT-GYÖRGYI AND JANE A. MCLAUGHLIN.

The extensive studies of our laboratory have led to the conclusion that the "living state" is a very specific physical state in which not all orbitals are occupied, leaving room for electronic motion. Proteins can be desaturated electronically by having single electrons taken out of them by "electron acceptors." The process of transferring an electron from one molecule to the other is called "charge transfer." Structural proteins have an inbuilt mechanism for their electronic desaturation. The kernel of this mechanism is the lysine side chain, which forms a schiff base and interacts with methylglyoxal and the second neighbor peptide link of the peptide chain.

Charge transfer greatly depends on the dielectric constant, ϵ, of the medium. A certain ϵ, can both inhibit and promote charge transfer. Certain transfers can thus take place only at a certain value of ϵ, making the dielectric constant an important biological parameter.

Alteration of blood clotting in Asterias forbesi *associated with a ciliate infection.* C. E. TAYLOR AND F. B. BANG.

Blood cells from a normal starfish aggregate to form clumps when exposed to glass. In 1970 and 1976, Childs and Bang found that whenever the starfish was infected by a ciliate parasite, the blood was milky and clumping was inhibited. Again this season there was a 100% correlation between ciliate infection and the inability of the blood to clot.

Efforts to induce clumping of cells from infected animals by ADP, convanavalin A, or cell lysate failed, even though the cell lysate clumping *in vivo* in normal clumpers and non-clumpers. Washing these cells in 0.5 M NaCl or resuspending them in cell-free supernatant from a normal clumper, or in various concentrations of calcium (0.01, 0.05, and 0.1 M) did not reconstitute clumping. However, when these cells were withdrawn into 0.1 M EGTA, washed in 0.02 M $MgCl_2$ in 0.5 M NaCl, then resuspended in 0.01 M $CaCl_2$ in 0.5 M NaCl or cell-free supernatant, clumping was reconstituted. EGTA-extracted cells from normal clumpers can also be washed and resuspended in a similar manner, reconstituting clumping.

Lowering the pH of blood taken from normal animals to pH 5 or lower by HCl will inhibit clumping. Boolootian showed that certain amino acids and sulphydryl reducing agents can inhibit clumping. In the present study, 0.01 M glutathione at pH 6.5 or lower or N-acetyl cysteine at pH 6.0 or lower prevented clumping of cells. L-methionine, L-cysteine, DTT and 2-mercapto-ethanol had no effects.

An interesting current finding was that blood withdrawn from sea stars immersed in a bowl containing 0.02 M $(NH_4)_2HPO_4$ in sea water for 1 to 2 hr had a milky consistency. Subsequent tests (macroscopic and microscopic examination) showed that the blood did not clot. While ammonia is the principal excretory product in most Protozoa, the relationship between these two phenomena—this and the failure of the blood to clot on glass—is unknown.

Spontaneous aequorin luminescence in Chaos carolinensis. D. L. TAYLOR, J. R. BLINKS, AND GEO. T. REYNOLDS.

Purified aequorin dissolved in chelex-treated distilled water was microinjected into *C. carolinensis.* The luminescence from single cells was detected with an image intensifier or photon counting system. Weak spontaneous luminescence was observed in the uroids of freely motile cells. The intracellular luminescence represented submicromolar free calcium based on the intensity of luminescence when *ca.* 10^{-6} M free Ca was injected into cells preloaded with aequorin. Intense luminescence was induced at the site of cellular damage caused by puncture with a micropipette. The luminescence was localized within a few microns of the plasmalemma and persisted for only a few seconds following damage. Electrical stimulation (*ca.* 10 volts/cm) resulted in luminescence at the anodal sides of cells. The intensity of luminescence was dependent on the extracellular calcium ion concentration and no luminescence was elicited in calcium-free solutions. Photon counting of single cells containing aequorin demonstrated a rather constant luminescence with pulses reaching intensities *ca.* 4 times the steady state. The above results are consistent with the pattern of electrical currents measured with an extracellular vibrating probe, where both a steady inward calcium current was detected in the uroid and spontaneous calcium-dependent currents were detected anywhere over the cell (Nuccitelli and Jaffe, 1977, *J. Gen. Physiol.,* **69**: 743). The giant amoeba either modulates contractile activity at concentrations of free calcium that appear to be submicromolar, or varies another parameter such as pH (Taylor, 1977, *Exp. Cell. Res.,* **105**: 413) to control contractile activity. Determination of the local intracellular pH should determine the relative importance of this latter parameter.

Supported in part by DOE DBER Contract EY-76-02-3120, NIH Grants AM 18111 and HL 12186.

Ca-K bi-ionic action potentials in squid giant axons. SUSUMU TERAKAWA.

The excitability of squid giant axons was examined under the bi-ionic condition in which the extracellular solution contained only $CaCl_2$ and the intracellular solution contained only K^+ salts (osmolarity adjusted by glycerol). An all-or-none action potential could be generated by electrical stimulation of the axon when it was bathed in a $CaCl_2$ solution of less than 20 mM, and internally perfused with a KF solution at a concentration of less than 100 mM. The amplitude of the action potential did not exceed 25 mV, and the duration was longer than 50 msec. Reducing the external Ca^{++} concentration to below 0.5 mM resulted in spontaneous, repetitive firing. Raising the Ca^{++} concentration from 2 to 20 mM reduced the amplitude and increased the duration of the action potential. The action potential was not suppressed either by 2 μM tetrodotoxin (TTX) or by 10 mM $CoCl_2$ applied externally.

Results obtained with voltage-clamp technique were as follows. There were two different conductance levels in the voltage-current relationship. The amount of the peak inward current was about 5 $\mu A/cm^2$. The curve of the reversal potential versus external Ca^{++} concentration had a Nernst slope when the holding potential was kept constant. The curve was relatively flat when measured with the holding potential adjusted so as to generate the largest inward current. Addition of 20 mM Na^+, Cs^+ or K^+ to the external solution were all equally effective in increasing the amount of peak inward current, and raising the reversal potential level. Addition of Na^+ or Cs^+, however, eliminated all inward current completely in the presence of 100 nM TTX. The enhancing effect of K^+ was not affected even by 1 μM TTX. It has been demonstrated that the bi-ionic action potential can be generated with K^+ as an internal cation, and that the sensitivity to TTX can be altered by external monovalent cation species.

Carbon monoxide evolution during photo-bleaching of chlorophyll in vitro. Robert F. Troxler, Larry Cooperstein, and Roger Lester.

The carbon monoxide (CO) cycle in the biosphere is incompletely understood. Recent measurements of ^{18}O abundance in atmospheric CO revealed five distinct CO species of different origin. One species, estimated to contribute 200 million tons of CO/year in the northern hemisphere alone, was postulated to arise from chlorophyll breakdown in senescent plants. Although the fate of chlorophyll in green plants is unknown, chlorophyll and heme are both derived from protoporphyrin IX in the porphyrin pathway and the conversion of mammalian heme to CO and bile pigment is well documented. The present study was undertaken to determine if CO is produced during chlorophyll photo-bleaching *in vitro*.

Chlorophyll a + b were extracted from spinach leaves and purified by solvent partition and thin layer chromatography on Silica gel G plates with toluene:ethyl acetate:absolute ethanol (8:2:1) as solvent. Chlorophyll-a was extracted from *Anabaena variabilis* (a cyanobacterium lacking chlorophyll-b) with 90% methanol, precipitated with dioxane and purified by chromatography on Sephadex LH-20 columns and on Silica gel G plates as above. The chlorophyll a + b mixture or chlorophyll a were dissolved in 175 ml absolute ethanol, placed in a 200 ml stoppered, jacketed flask (gas phase = 25 ml) and irradiated with a 600 watt photographic light for 75 to 90 min at 10° C. Chlorophyll photo-bleaching (loss of absorbance at 660 nm) and CO evolution in the gas phase (Beckman 315A IR analyzer; CO conversion to CO_2, alkali trapping and titration of residual alkali) were determined.

When irradiated in air, chlorophylls a + b were converted to red pigments (absorption bands at 575 and 530 nm) with concomittant evolution of CO. The molar ratio of chlorophylls photobleached to CO evolved was approximately 2:1. Photo-bleaching of chlorophylls a + b was inhibited 80% under a nitrogen atmosphere, and 100% inhibition was observed after conversion of chlorophylls a + b to pheophytins a + b (by removal of magnesium from the macrocycle) after adding 1 ml of concentrated HCl to the ethanol solution prior to irradiation. Comparable results were observed with purified chlorophyll-a alone in air, under nitrogen, or in air as the pheophytin-a derivative.

These results indicate that molecular oxygen and a metal (magnesium) in the chlorin ring are necessary for chlorophyll photo-bleaching, and that CO is evolved in this process. This is consistent with the existence of a species of atmospheric CO appearing in the autumn and presumed to arise from chlorophyll breakdown in senescent plant tissues.

Supported in part by NSF Grant PCM76 24139 and NIH Grant AM HD 17847.

Initiation of RNA synthesis by isolated chick embryo nuclei. Steven Wood and Susan Astrin.

Nuclei of chick embryos can initiate RNA synthesis *in vitro*. This step of RNA synthesis can be differentiated from elongation by using purines that have a sulfer atom substituted for an oxygen atom in the γ-phosphate. Any RNA chain that is initiated will have a thio moiety. These RNAs are isolated by binding them to a mercury-agarose column and can be eluted in a step gradient of dithioeyrthritol. This is the method of M. Smith, A. Reeve, and R. Huang (*Cell*, in press) with some modification.

Nuclei were prepared by mincing four 9 to 11 day old chick embryos and homogenizing in 2 volumes of 2.3 M sucrose. The homogenate was diluted to 100 ml with sucrose and spun for 3 hr at 30,000 rpm in a SPINCO #30 head. After resuspending the pellet in 3 ml of freezing solution which contained 25% glycerol, 5 mM Mg acetate, 50 mM HEPES pH 8, and 10% BSA, the nuclei were stored at −70° C.

The reaction mixture contained 1 μl 1 M $MnCl_2$ 10 μl 1.5 M KCl, 4 μl 1 M tris, 1.5 μl 40 mM s-ATP, 1.5 μl 40 mM s-GTP, 0.74 nM ^3H-UTP 35 Ci/mM, 1.5 μl 40 mM CTP and 10^7

nuclei. The volume was brought up to 85 μl with H_2O. After 45 min at 37° C, the reaction was stopped by the addition of 1 ml of 10% SDS, in 0.01 M tris pH 7.9, 0.01 M EDTA, 0.1 M NaCl (TNES) and applied to a mercury-agarose column equilibrated with TNES. One milliliter fractions were taken as the column was washed with 10 ml of TNES and then with 2 mM dithioeyrthritol in 10 ml TNES. Fractions were precipitated by 0.5 M HCl, transfered to a glass fiber filter, dried, and counted in 1 ml of ECONOFLOUR.

A plot of cpm versus fraction number revealed two distinct peaks. The RNA eluted in the TNES wash were those chains elongated *in vitro.* RNA released by dithioeyrthritol were the molecules initiated during the reaction. We found that 5 to 7% of the RNA synthesised in the second peak. Chick nuclei are capable of initiating at moderate rates. When the reaction was run with ATP and GTP in place of the thio purines, some RNA was eluted in the dithioeyrthritol step gradient. This background could not be lowered by increasing the EDTA concentration of the TNES buffer. A phenol extraction of the reaction mixture followed by an ethanol precipitation removed this nonspecific binding.

This method provides a very sensitive assay for the expression of genes *in vitro.* The mercury-agarose column purifies the initial transcripts, which can be hybridized to specific probes using Southern blots.

This work was supported by PHS Grant #00265 to the Physiology course.

Comparison of three methods for measuring orthophosphate concentrations in natural waters. MARK N. WHITE, MICHELLE DIONNE, AND BRUCE PETERSON.

Comparison of three methods for determining orthophosphate concentrations were carried out during the spring of 1978. Chemical (soluble reactive phosphorus, referred to as SRP), radiobiological, and enzymatic techniques were used. The SRP method is based on the spectrophotometric determination of phosphate by the molybdenum blue reaction. The radiobiological analysis assesses the concentration of inorganic phosphate based on a kinetic analysis of $P^{32}O_4$ uptake in ambient and phosphate enriched samples. The enzymatic method is based on the relative inhibition of the phosphatase enzyme by phosphate in the water sample. The enzymatic method is being developed as an alternative to the SRP which appears to overestimate phosphate concentrations. Cape Cod, Massachusetts ponds and a batch culture were used in the comparisons. The batch culture simulated conditions of a spring bloom, *i.e.,* high initial phosphorus concentration and low algal biomass.

Only the SRP and enzymatic techniques were used on water samples from beneath the ice in Cape Cod ponds. February 16, Ice Pond: 0.03 (enzymatic) *vs.* 0.10 (SRP). March 2, Shivericks Pond: 0.03 (enzymatic) *vs.* 0.56 (SRP). March 31, Siders Pond: 0.085 (enzymatic) *vs.* 0.47 (SRP). Values given by the enzymatic technique were 33, 13, and 18%, respectively, of those given by the SRP method.

Water taken below a sewage outfall on the North River (Rockland, Mass.) was used for the batch culture. The culture was maintained at constant temperature and light. The depletion of dissolved inorganic phosphorus and increase in chlorophyll was followed by sampling the culture once each day. In eight days the chlorophyll a concentration rose from 8.81 to 69.42 mg/m^3. Values for orthophosphate concentrations in the culture given by the SRP, enzymatic, and radiobiological methods (as μg-at P/l) follow: day 1–2.67, 1.69, and 0.50, respectively; day 3–1.11, 0.92, and 0.15; day 6–0.76, 0.58 and 0.15.

In both the batch culture and pond studies, the enzymatic method consistently gave lower values than the SRP method. The values given by the enzymatic method, however, ranged from 13 to 81% of the SRP. Furthermore, in the few culture samples obtained, the radiobiological method gave values well below those given by the other two methods.

This work was supported in part by a scholarship from the Jessie Smith Noyes Fellowship.

Aging and cataract in dogfish (Mustelus canis) *eye lenses.* SEYMOUR ZIGMAN AND TERESA YULO.

Dogfish, due to their comparatively long life span (up to 50 yrs), are ideally suited for studies of lens aging. Although their shape, texture, environment, role in visual accommodation, and certain chemical features (*i.e.*: pigment lack; presence of 0.3 M urea) differ from those of humans, the structural proteins, cell morphology, and the process of growth and differentiation are quite similar. The differences may represent adaptations to an aquatic rather than a terrestrial environment.

In this study, changes in the size distribution and the nature of the subunits of the lens crystallins were studied as a function of age. As dogfish grow from a length of 10 to 15 in. up to 45 to 50 in., the lens weight increases from 100 to 1500 mg, a process that takes 40 to 50 yr. The dry weight is constant at about 50% of the wet weight, and thus these lenses are dehydrated compared to human lenses.

The preponderance of protein chains within the soluble (100,000 g supernatant), high molecular weight colloid (100,000 g pellet), and insoluble (fiber cell membrane-rich, 12,000 g sediment) fractions have 20,000 to 30,000 daltons molecular weights, using SDS-polyacrylamide gel electrophoresis for assay. With aging, subunits between 30,000 and 80,000 daltons accumulate very slowly in the soluble, but more quickly and extensively in the aggregated fractions. The crosslinks of the heavier subunits are covalent, but are not yet characterized. Purification of the fiber cell membranes by extracting them with water and 8 M urea reveals two chains (45,000 and 50,000 daltons) which represent membrane proteins, but chains of 20,000 to 30,000 daltons still predominate. The crystallins of this basic size appear to be integral components of the membranes.

Molecular aging in the dogfish lens thus is the result of the aggregation of smaller subunits into larger ones, and their incorporation into fiber cell membranes as they accumulate with age. Why dogfish lenses remain transparent in spite of much light scattering from levels of aggregated proteins of over 50% (a situation which occurs in human lenses only with cataracts) must still be explained.

Support: The National Eye Institute and The Marvin Pledger Fund (University of Rochester).

Continued from Cover Two

of biological journal titles is that published each year by Biological Abstracts (biosis List of Serials; most recent issue, 1976). Foreign authors, and others who are accustomed to use The World List of Scientific Periodicals, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. at £0.65 or $1.75) useful, since it sets out the World List abbreviations for most biological journals with notes of the USASI abbreviations where these differ. Chemical Abstracts publishes quarterly supplements of additional abbreviations. The following points of reference style for The Biological Bulletin differ from USASI (or modified World List) usage:

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Volume 155 Number 3

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Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
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THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $8.00. Subscription per volume (three issues), $22.00, (this is $44.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

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Vol. 155, No. 3 December, 1978

THE

BIOLOGICAL BULLETIN

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BIOLUMINESCENCE AND OTHER RESPONSES SPREAD BY EPITHELIAL CONDUCTION IN THE SIPHONOPHORE *HIPPOPODIUS*

Reference: *Biol. Bull.*, **155**: 473–498. (December, 1978)

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Conducting epithelia are known to play an important part in the behavior of several groups of animals including hydrozoan coelenterates and amphibian larvae (reviewed by Mackie, 1970; Spencer, 1974), and skin conduction has recently been reported in pelagic tunicates and ascidian tadpoles (Bone and Mackie, 1975; Mackie and Bone, 1976).

These epithelia include both simple, non-muscular sheets of cells and myoepithelia. It has been shown in a few cases that the cells are electrically coupled (Roberts and Stirling, 1971; Mackie, 1976), which suggests that impulses propagate from cell to cell by direct current flow. Even where direct evidence of coupling is lacking, the presence of gap junctions points to the same conclusion.

While hydrozoan conducting epithelia are typically either themselves contractile or provide a pathway for activation of a muscle (see Table I in Mackie and Passano, 1968), the siphonophore *Hippopodius* presents an unusual wealth of epithelially-mediated responses including, in addition to muscular involution, luminescence, blanching (temporary increases in opacity) and discharge of secretory material (Mackie, 1965; Mackie and Mackie, 1967; Mackie, 1976). This paper presents new results on these phenomena and their control. A general account of the anatomy and behavior will first be given.

Hippopodius is a colony consisting of a stem from which are budded a series of medusoid members, the nectophores, as well as other, polypoid, members

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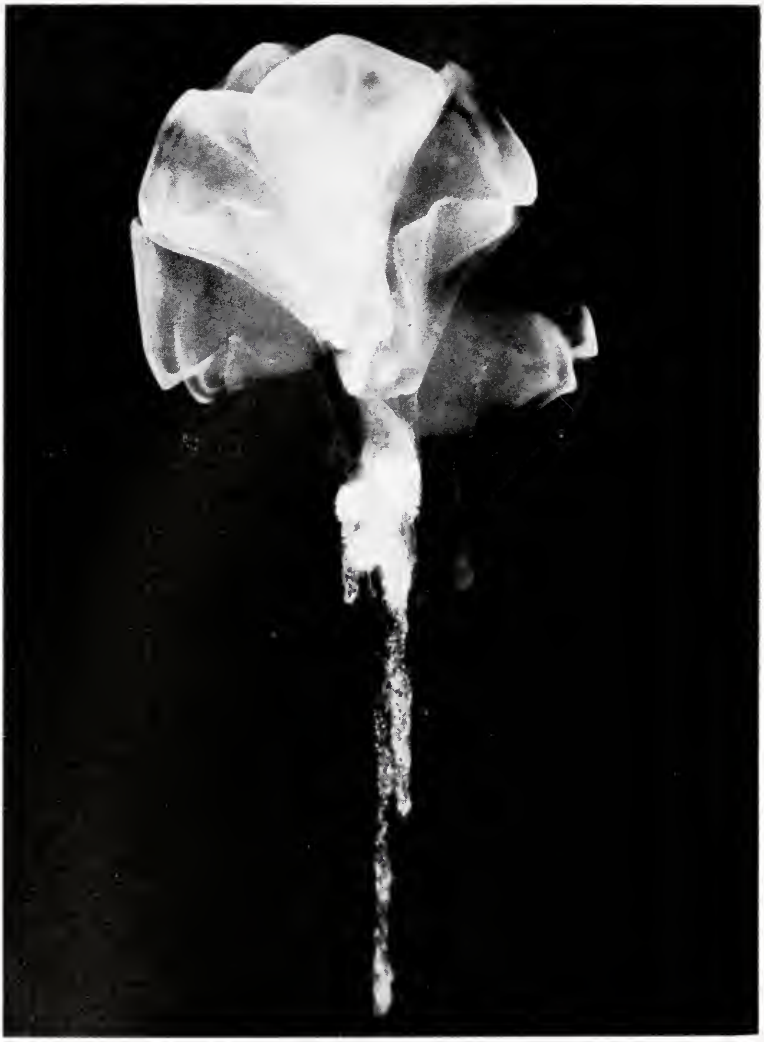

FIGURE 1. Photograph of a living *Hippopodius*. The stem and appendages are partially retracted into the central space. The upper nectophores are more strongly blanched than the lower. The lowest nectophore (on the right) is transparent except for the marginal "prongs". Actual size of the colony is about 7 cm.

(Carré, 1968) ; the nectophores (also called nectocalyces) are the largest individuals and the only locomotory ones. The group of nectophores encloses a central space into which the stem and gastozooids can be withdrawn (Figs. 1, 2). There is no float, but the gelatinous mesogloea of the nectophores provides flotation (Jacobs, 1937).

The nectophores lack tentacles, sense organs, gonads and manubrium but resemble solitary hydromedusae in their locomotory neuromuscular organization.

Nutrients are conveyed to them by a canal from the stem which splits into branches upon entering the subumbrella. One of these, the ventral canal, is the site of an epithelial gland, the *rete mirabilis* or rete. Nectophores can swim using their circular, striated muscle, or roll up the margin (involution), using the radial system of smooth muscle fibers. Marginal nerve rings are present as in hydromedusae, but there is no general nerve plexus over the sub- or ex-umbrella and there are no nervous connections between the marginal nerve centers of the different nectophores in the colony (Mackie, 1965).

Considered as a medusoid, the nectophore is highly asymmetrical compared with free medusae (Figs. 1, 2, 14). The mesogloea is concentrated dorsally, while the subumbrella cavity is displaced ventrally. The outer or dorsal surface is a convex dome, the inner, ventral side being more concave. These features allow the nectophores to fit together into an interlocking structure with smooth sides and with the swimming jets directed backward. The exumbrella surface is generally

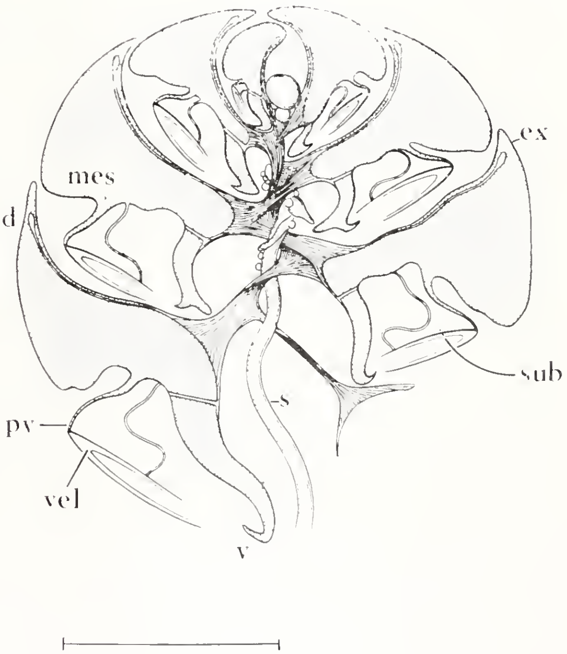

FIGURE 2. Internal morphology (after Chun, 1897). The "axis" of the colony is defined by the stem(s) which hangs in a central space surrounded by nectophores. Branched stem processes join the nectophores together, attaching to their axial surfaces. "Top" and "bottom" refer to aspects of the colony seen in its normal orientation, as shown here. "Dorsal" and "ventral" poles of the nectophore are indicated for the bottom nectophore on the left (d, v); the exumbrellar surface (ex) is the entire outer surface of the nectophore; the subumbrellar cavity (sub) is the inner chamber whose muscular walls produce the locomotory water jet. Other symbols: mes = mesogloea; pv = pseudovelum; vel = velum. Scale is 1 cm.

smooth except for a ridge bearing four prominent mounds ("prongs") encircling the upper rim of the subumbrellar cavity, and two ventral projections, or "horns".

The exumbrellar epithelium is a fragile, thin layer, one cell thick in all parts. It is thicker over the horns than elsewhere. It is partially syncytial (Mackie, 1965), but no physiological differences have been found between the syncytial and non-syncytial regions.

Freshly collected specimens of the siphonophore float or swim freely in the aquarium, but after a few hours most sink to the bottom. Jacobs (1937) suggests that they can regulate their buoyancy. All the nectophores make swimming movements but, except for the bottom two, which are responsible for locomotion, their activities do no more than create water movements within the center of the colony. As noted by D. A. Boag (cited by Mackie, 1964), swimming is not synchronized, although activity beginning in one nectophore may create a mechanical disturbance which causes others to become active. As in hydromedusae (Romanes, 1876; Passano, Mackie and Pavans de Ceccatty, 1967) the swimming rhythm is presumably independently generated by nervous pacemakers in the margin of each nectophore. Although there are no ocelli, *Hippopodius* aggregates in lighted regions of the tank (Mackie and Boag, 1963). This suggests that the pacemaker neurons controlling swimming are themselves photoreceptors, as in the jellyfish *Polyorchis* (Anderson and Mackie, 1977).

There are four, separate, direct effector responses to nectophore stimulation that are conducted through the colony, at least in part, by epithelial conduction (Mackie, 1965).

Involution of the margin (a response resembling "crumpling" in solitary hydromedusae) follows stimulation of any part of the exumbrellar surface; the epithelium is excitable, and conducts impulses to the margin where the radial muscles responsible for involution are located.

Undamaged specimens which have not recently been stimulated are transparent, but tactile or electrical stimulation causes the nectophore to become opaque; this "blanching" (Korotneff, 1884) is due to light-scattering granules which appear extracellularly in the mesogloea adjacent to the exumbrellar epithelium Kölliker, 1853) and are seen under the microscope as dense, spherical particles in the range 0.1 to 1.5 μm (Mackie and Mackie, 1967). Intact epithelium is necessary both for the spread of the blanching response and for the restoration of transparency, which occurs after a delay of several minutes. Mackie and Mackie (1967) proposed that impulses propagated in the epithelium trigger blanching. Tentative evidence was found for a role of Ca^{++} in the response and it was suggested that Ca^{++} activates the transformation of a mesogloeal protein between dispersed and aggregated configurations.

Seen in the dark, *Hippopodius* luminesces briefly when exposed to the same sorts of stimuli that provoke blanching and involution. Dubois (1914) has stated that this "magnificent bluish illumination" comes from the epithelial cells. Mackie and Mackie (1967) found that luminescence requires an intact exumbrellar epithelium and they proposed that photogenesis is intracellular and triggered by impulse conduction. Nicol (1958) obtained photometric records from *Hippopodius* and the closely related *Vogtia*. The chemical basis of the luminous reaction remains unknown but, as reviewed by Morin and Hastings (1971); Cormier, Hori, Karkhanis, Anderson, Wampler, Morin and Hastings (1973); Anderson, Charbonneau and Cormier (1974); Morin (1974); and Blinks, Prendergast and Allen (1976), a calcium sensitive photoprotein like aequorin has been found in all bioluminescent coelenterates so far studied.

The fourth effector response associated with epithelial conduction occurs in the rete, a patch of giant endoderm cells clustered around ramifying branches of the ventral radial canal. The cells are specialized for protein secretion, but the chemical identity and biological role of the product remain uncertain. The rete cells are electrically coupled and connected by gap junctions. Impulses propagate through the tissue at about 10 cm/sec. When stimulated repetitively, the potentials associated with secretion sum and facilitate. The cells swell up and release secretion product; this process is Ca^{++}-dependent (Mackie, 1976).

These four responses tend to occur together under similar circumstances, for instance, when the nectophore surfaces are stimulated by abrupt contact. It has been suggested (Mackie and Mackie, 1967) that they have a common protective significance, the involution protecting the marginal locomotory centers, and luminescence and blanching serving to confuse, blind or alarm predators. The role of the secretion is not known but it may also be defensive. Spread of these responses through the colony accompanies colony-wide spread of epithelial impulses. An impulse may fail to spread beyond the bounds of a single nectophore but, with repetitive stimulation, all parts are rapidly invaded. At the same time, the stem is withdrawn, swimming ceases, and the colony remains in this defensive condition for periods up to several minutes, depending on the intensity with which it has been stimulated.

MATERIALS AND METHODS

Specimens of the siphonophore *Hippopodius hippopus* Forskal were collected during spring, with a hand net, at the surface, in the bay of Villefranche sur Mer (France), where they occurred in the plankton with highly variable abundance from day to day. Stored at 15° C, in water changed daily, they remained healthy for a few days. Best maintenance was achieved when they were kept in freshly obtained, uncontaminated sea water.

Nectophores were isolated by cutting the stem which links them to the colony and placing them in a glass dish with flat, parallel sides and the bottom coated with a layer of transparent, polymerized resin (Sylgard 184, Dow Corning). They were immobilized either by pinning through the exumbrella or by restraining them within a corral of pins. Most of the records were made at room temperature (about 20° C) but, in some cases, a water-cooled microscope stage was used to keep the observation chamber at about 16° C. Before experiments involving stimulation of luminescence or blanching, the preparations were allowed to rest in darkness for at least 10 min.

The behavioral responses of the nectophores were analyzed using photometric procedures (Fig. 3) and records were made either on a storage oscilloscope (Tektronix 5015) or on chart recorders (Hewlett Packard 7402 A).

Bioluminescent emissions were detected by a RCA 1P21 photomultiplier (PM)

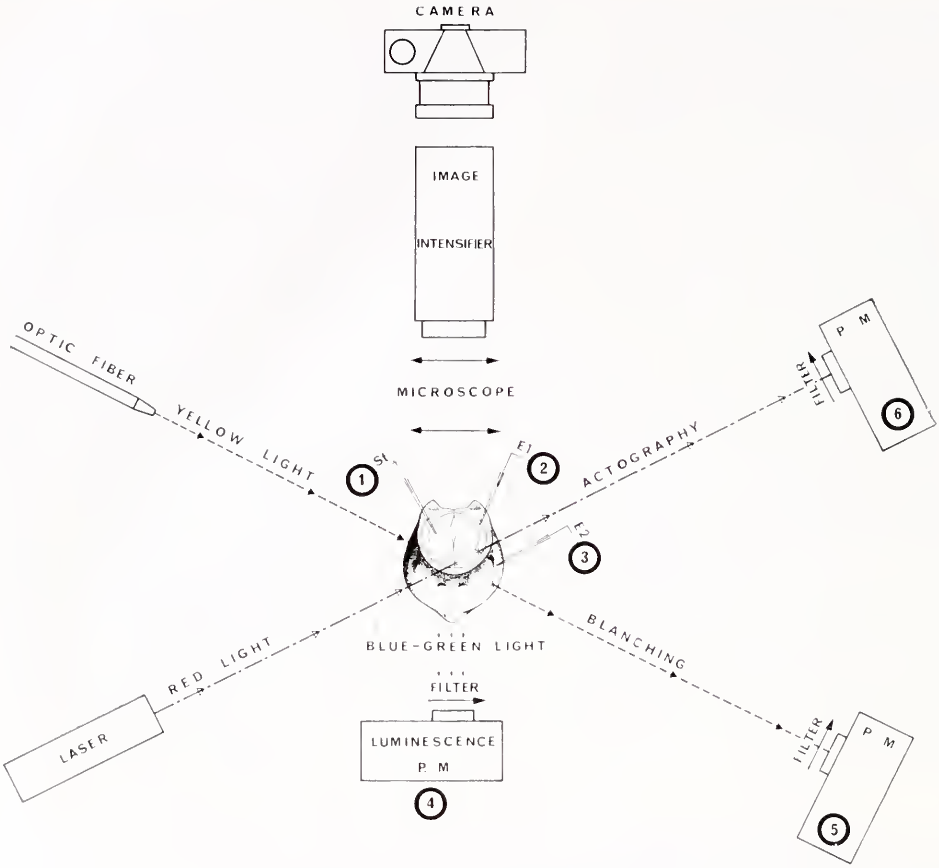
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FIGURE 3. Schematic drawing of the experimental set up. Suction electrodes (1), (2), (3) allow stimulation and recording of electrical events. Luminescence is detected with a photomultiplier (4), while the sites of emission are observed or photographed through a microscope and image intensifier. A beam of yellow light crosses the exumbrella, and changes in transparency are measured with a photomultiplier (5). A beam of red light emitted by a laser crosses the velar region, making possible the detection of movements with a photomultiplier (6).

located below the preparation, and restricted to the blue range with a low pass filter (MTO 529b). In some preparations, luminescence was recorded from small areas of the surface of the animal, using one or two 1 mm diameter optic fibers leading to a EMI 9636 PM.

Actography was done by using a continuous laser (Spectra-Physics He-Ar, 1 mW). The red beam (6340 Å) of coherent light, attenuated by a netural filter (T: 20%), was set to cross the muscular ring of the pseudovelum. The diffraction image thus obtained was enlarged with a lens and delimited by a diaphragm so as to fit the diameter of the window of the PM, itself adjustable with another diaphragm. A narrow band filter (MTO 7625a) restricted the PM sensitivity to the laser light only.

Changes in transparency have been recorded in some instances with this laser device, since blanching attenuates the light by diffusion. Nevertheless, a larger beam of normal (non-coherent) light proved to be more sensitive for blanching measurements, and it was less affected by movements. The light emerging from a light pipe, illuminated by a battery powered quartz-iodine lamp, was narrowed with a condenser lens to a beam of about 3 mm diameter and set to cross the exumbrella. An orange or yellow-green filter was used to obtain monochromatic light; an identical filter was placed on the window of the PM. It was thus possible to get simultaneous records of bioluminescence, blanching and muscular movements (Fig. 3).

Photography of the preparation by its own light was done through a Wild stereomicroscope. Owing to the brief duration and weakness of the light emission to be recorded, an image intensifier (Thomson CSF, three stages, gain: 50,000), was interposed in front of the camera. Its enhanced image was recorded on fast panchromatic (Ilford HP400) film; the exposure time, of 1 to 10 sec was recorded together with the photometric measurements. In some cases, the luminescent image was recorded with a TV camera and tape recorder (Sanyo VTC 7100) to allow frame by frame analysis of the flashes.

Electrical activities were generally recorded with suction electrodes made from drawn-out polyethylene catheter tubing and held by mechanical micromanipulators. The signals were amplified with Tektronix AC or DC high gain preamplifiers allowing frequency filtration. Mechanical stimulation of the nectophores was done by gently touching them with a fine plastic tube, such as a suction electrode. For electrical stimulation, monophasic pulses generated by a Grass S 44 stimulator were fed through an isolation unit to metal electrodes

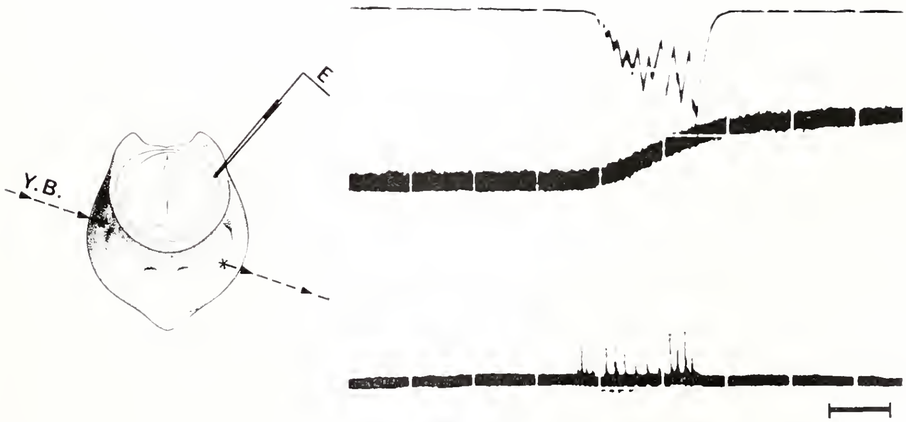

FIGURE 4. Spontaneous activities. Correlation of bioluminescence (upper line, downwards) and blanching (middle line, opacification upwards) with epithelial signals (lower line) recorded with electrode E on the velar region. Y.B. shows the beam of yellow light used to measure transparency. Scale is 2 sec.

or, more often, to a suction electrode attached to the surface of the animal or resting gently against it.

Experimental operations on the nectophores included surgical interventions such as ablation of the nerve ring, immobilization of a dissected piece of tissue held to the Sylgard base with very fine cactus spines, and cutting of parts of the nectophore. Fragments of nectophores were fixed for electron microscopy in 6% glutaraldehyde in a 0.4 M cacodylate buffer pH 7.4, embedded in epon, cut with a LKB microtome and observed with a Philips EM 300 microscope.

RESULTS

Spontaneous events

Isolated nectophores remain capable of all the behavioral activities described in the colony. Usually, a nectophore which has been detached from the colony and immobilized in the observation chamber is opalescent (blanching having occurred during manipulations) and completely motionless. After 10 to 20 min, transparency is recovered again, except at the spots where pins have pierced the epithelium; the prongs and the horns are the last to lose their opacity.

The first "spontaneous" movement to be observed is an involution of the margin, followed by a slow relaxation. Then, other involutions may occur. Independently, isolated flashes or short flickering series of flashes are emitted (Fig. 4), again without any external stimulation. Combined records show blanching occuring together with each luminous event. A period of quietness follows, which usually lasts 10 to 20 min. Then swimming begins, in bursts of rhythmic activity alternating with periods of rest of 10 to 30 sec. The duration of swimming bouts steadily increases. The beating rhythm is very regular, about 4/sec at 20° C (Fig. 5). Our actographic technique, which measures changes in the diffraction pattern of the laser beam, has an extreme sensitivity towards minute displacements, but the shape and the amplitude of the curves are not significant; they change considerably according to the orientation of the beam. It is still noteworthy that under the same conditions, records of successive beats

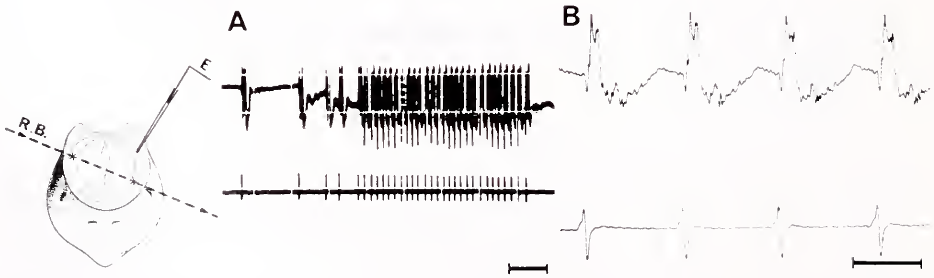

FIGURE 5. Swimming recorded actographically (upper line) and electrically from the velum (lower line). R.B. indicates the laser beam of red light used for actography; swimming typically begins irregularly and becomes rhythmic, ceasing abruptly. Scale is .5 sec for figure A and 200 msec for figure B.

are absolutely identical. Bioluminescence has never been observed during swimming.

Specific electrical signals are related to these activities. One type of potential is associated with involution. Another accompanies each swimming contraction (Fig. 5). Each flash is associated with a potential even in the case of multiple flashes where summation occurs (Fig. 4). But while the potentials associated with muscle contraction are best recorded on the marginal ring, those associated with bioluminescence spread over the whole exumbrellar surface and can be recorded on every part of it. Other spikes have been recorded on the nerve ring. They are without any obvious behavioral correlates, and show erratic patterns, consisting of isolated pulses or short bursts, the potentials usually being of small amplitude. Some of these events may be homologous to known pulses in hydromedusae (see Passano, 1973).

Effect of stimulation

It appears that the effector responses of the nectophore are organized within two general action systems, each with its own conduction pathways.

Involution, bioluminescence, blanching and secretion by the rete gland may result from mechanical or electrical stimulation applied *anywhere* on the surface of the exumbrellar epithelium. These stimuli never induce swimming; on the contrary, swimming stops when involution occurs.

The *swimming system* is independent. From the histological relationships and by analogy with solitary hydromesudae, it can be assumed that the swimming rhythm originates in the marginal nerve ring. Since there is no subumbrellar nerve net, transmission to the striated muscle sheet must occur locally in the vicinity of the nerve ring and conduction within the sheet must be myoid. Swimming is blocked by the addition of Mg^{++}, which presumably blocks the neuromuscular junctions. The circular muscles of the velum act together with those of the pseudovelum (the flexible margin of umbrella) and can be considered as a part of the same functional unit.

The other system involves epithelial conduction. Following stimulation any-

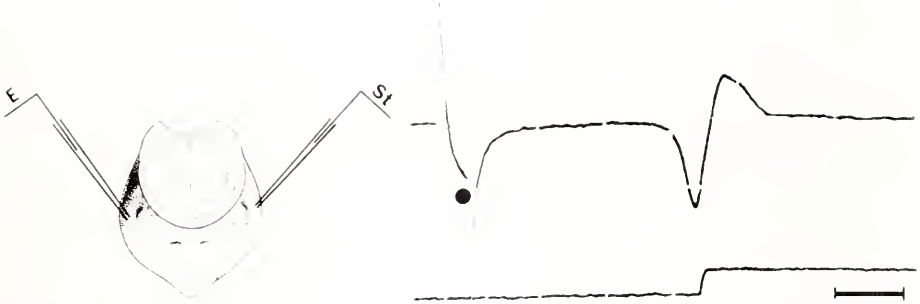

FIGURE 6. Epithelial potential recorded from the exumbrella with electrode E, after stimulation at St. The dot indicates the stimulation artifact. The amplitude calibration on the lower trace is 200 μv and the time scale 10 msec.

where on the exumbrella, a potential can be recorded at any place on the exumbrellar surface (Fig. 6). Its propagation is non-decremental; conduction time corresponds to the distance between stimulating and recording electrodes. The speed of propagation is appoximately 10 cm/sec at 22° C.

Involution

A single electrical shock above threshold leads to involution. The muscular components involved are two sets of smooth muscle fibers orientated radially, one in the velum and one in the subumbrellar endoderm of the pseudovelum (Fig. 7). Contraction of the first set of fibers causes the whole velum, which is like an iris diaphragm, to dilate and, seen in radial section, to curl outward. Contraction of the second set causes the pseudovelum to roll inward, carrying the curled velum within it. Impulses propagated through the exumbrellar epithelium normally cause both sets of muscles to contract in the involution response although, after repeated stimulation, one component may fail before the other (Fig. 8). The response amplitude is graded according to the number of impulses generated. Stimulation of the subumbrella is also effective.

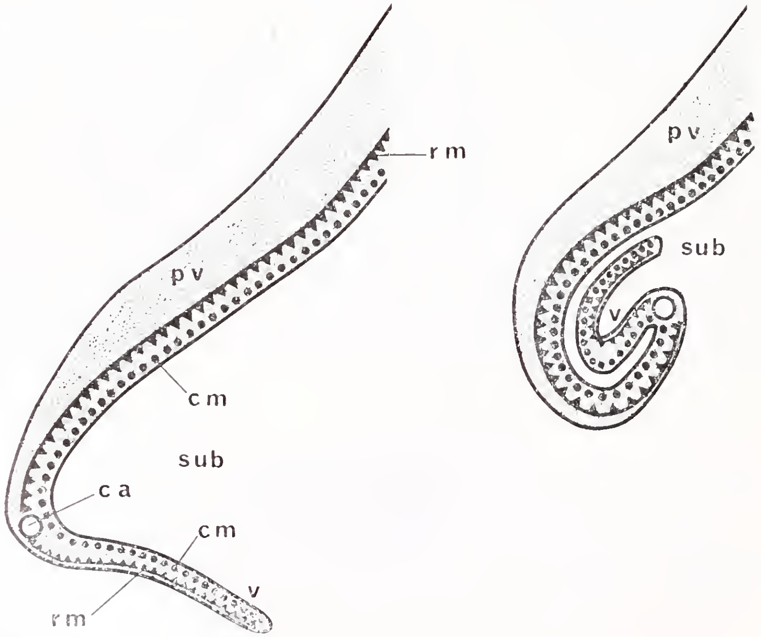

FIGURE 7. Schematic radial section through the contractile region of a nectophore, left at rest and right after involution. Involution is caused by the contraction of the two sets of radial muscle (rm) of the velum (v) and pseudovelum (pv), respectively. The circular muscle (cm) of the swimming system is not active during involution; ca represents the ring canal and sub the subumbrellar cavity.

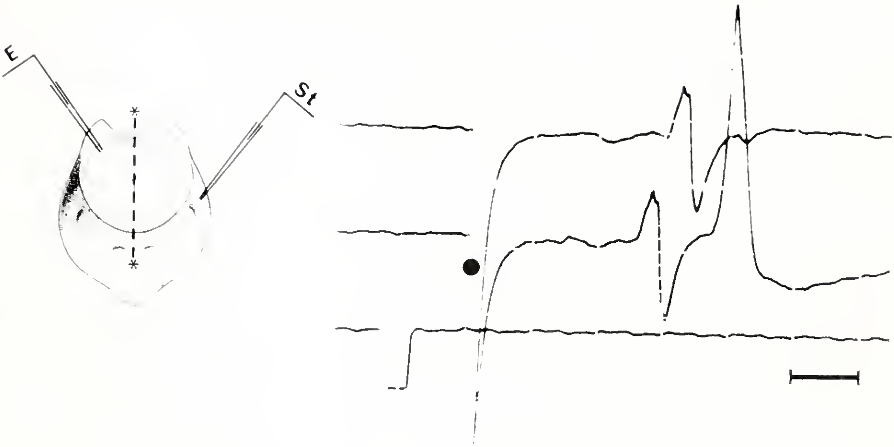

FIGURE 8. Experiment demonstrating the conduction across a "bridge" of exumbrellar epithelium. An incision (dashed line between asterisks) prevents conduction in the muscular regions. The experiment also illustrates the two components of involution. In the first trace, only the velar radial muscles responded. In the second trace, those of the pseudovelum responded immediately afterwards. The dot indicates the stimulation artefact; the lower line shows the 200 μv voltage calibration. Time scale is 10 msec.

Bioluminescence

"Ice blue" in color, often surprisingly strong, bioluminescence accompanies involution and, like it, is stimulated by mechanical or electrical shocks (Fig. 9). The basic response is a single flash, highly variable in duration (40–400 msec) and characterized by a fast ascending slope and a longer decay of the first order; double or multiple flashes also often occur and several flashes may lead to a rapid flickering post discharge (Fig. 13). Glow has never been observed.

Repetitive stimulation at 5 or 1 Hz, just above threshold, evokes one to one responses. Series which comprise 10 to 40 flashes show first a period of facilitation, followed by a more or less fluctuating plateau (Fig. 9). This may cease abruptly or decay gradually. For bioluminescence to be re-evoked, stronger stimulation or a period of rest is necessary.

One to one responses follow an acceleration of the stimulation frequency up to about 10 Hz. In addition to facilitation, partial summation is then apparent.

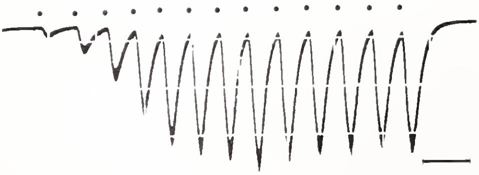

FIGURE 9. A series of shocks at lower frequency (dots) evokes a facilitating series of flashes. Scale is 1 sec.

Post discharges occur with stronger stimuli consisting of brief bursts at high frequency. When such stimuli are repeated, successive post discharges get longer and brighter, involving more and more cumulative flashes. They often lead to the climax of an explosive response, in which flashes flicker at high frequency (10 per second) for several seconds. After such intense events, which remind the "frenzy" responses observed by Buck (1973) in *Renilla,* the system is nearly exhausted. Nevertheless, after 5 to 20 min of recovery, the system is again responsive.

The effectiveness of electrical stimulation has proven to be highly variable in different individuals. This is probably due to the fragility of the epithelium which, if damaged by the electrodes, can be excited only by increased stimulation.

An epithelial potential is associated with each single flash as well as with each peak of a multiple response (Fig. 10). These pulses remain constant in shape and amplitude regardless of the brightness of the associated flash. Response latencies vary depending on the placement of the electrodes.

Initially, at least three consecutive epithelial pulses are required to elicit the first component of the luminous response (Fig. 10). Thereafter, one to one responses occur when the stimulating shocks, and thus the epithelial pulses, are

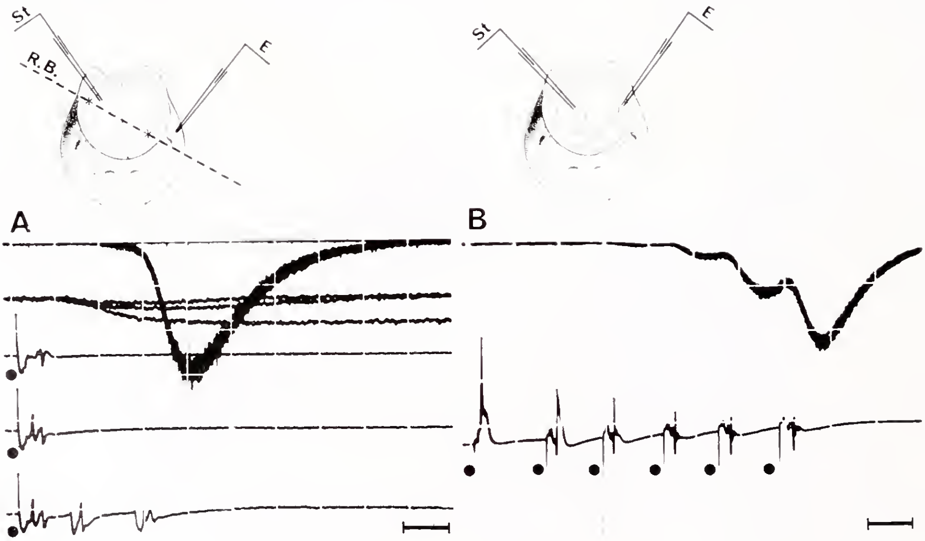

FIGURE 10. Induction of luminescence. On left, A is a simultaneous record of luminescence (upper line), involution (actographic record, next below) and epithelial impulses (three lower records, successive sweeps about 4 sec apart). Single epithelial impulses produced perceptible degrees of involution, but no flash occurred until three epithelial pulses were evoked. Scale is 50 msec. On right, in B, a series of shocks evoked a corresponding series of epithelial pulses (lower trace). After the third one luminescence was evoked; each subsequent spike was associated with a peak of the luminous response. Summation and facilitation are apparent; the latencies are not significant; amplitude changes in the recorded electrical signal are due to movement of the tissue with respect to the tip of the recording electrode. Scale is 0.2 sec.

separated by less than 3 to 5 sec. Otherwise, two or three pulses are needed before again obtaining a luminous response.

The post discharge volley of epithelial pulses, as well as corresponding flashes observed after intense stimulation, often show an inherent rhythmicity.

Sites of emission

Although the transparency of the nectophore and the shortness of the flashes make observation difficult, it appears clear that the light arises from the most external layer of the exumbrella. The velum, the subumbrellar surface and internal structures such as the endodermal canals crossing the mesogloea never luminesce. Removal of part of the exumbrellar epithelium results in loss of the ability of this region to luminesce.

Nevertheless, the exumbrellar surface does not respond as a whole to stimulation, whatever the placement of the electrodes. What is more, the active area moves its boundaries from one flash to the next. Usually, the response originates in the horns. Successive flashes of increasing intensity extend the active territory along two symmetrical, curved and narrow paths, which soon fuse, forming a sort of horseshoe around the margin. Then, with further flashes, the active territory moves toward the bell-shaped dorsal face of the nectophore. Illumination is then particularly strong beside the horns and around the marginal prongs. The apex of the dome is invaded only during paroxysmic responses. With the last flashes, which become weaker and weaker, the active territory either shrinks quickly to the originally active zones or moves to parts of the horseshoe region which had not been fully exploited previously. Photographic records through the image intensifier (Figs. 11, 12) required the integration of several successive flashes for correct exposure. Nevertheless, moving boundaries and increase of active surface are well demonstrated in correlation with the facilitation which occurs during successive series of responses.

Observed during a single flash, the active area, whatever its localization and dimensions, gives the impression of being illuminated by a wave. In the case of a rapid succession of flashes, this wave rebounds and whirls, often switching alternatively to the left and the right sides of the nectophore. This asynchronous illumination of the area of activity is clearly related to the duration of the flashes and their waveform.

Blanching

Blanching is also a property of the exumbrella, but involves both the epithelium and the underlying cortical layer of the mesogloea, where the light-scattering granules actually appear.

Blanching first appears as a local response at the point of application of the electrical or mechanical stimulus, then spreads over the whole exumbrella as a wave. The first generalized response of opacification is the strongest one. It takes up to 10 sec to build exponentially to a stable level. With further shocks, blanching responses get smaller and smaller, apparently reaching exponentially the maximum level of opacification.

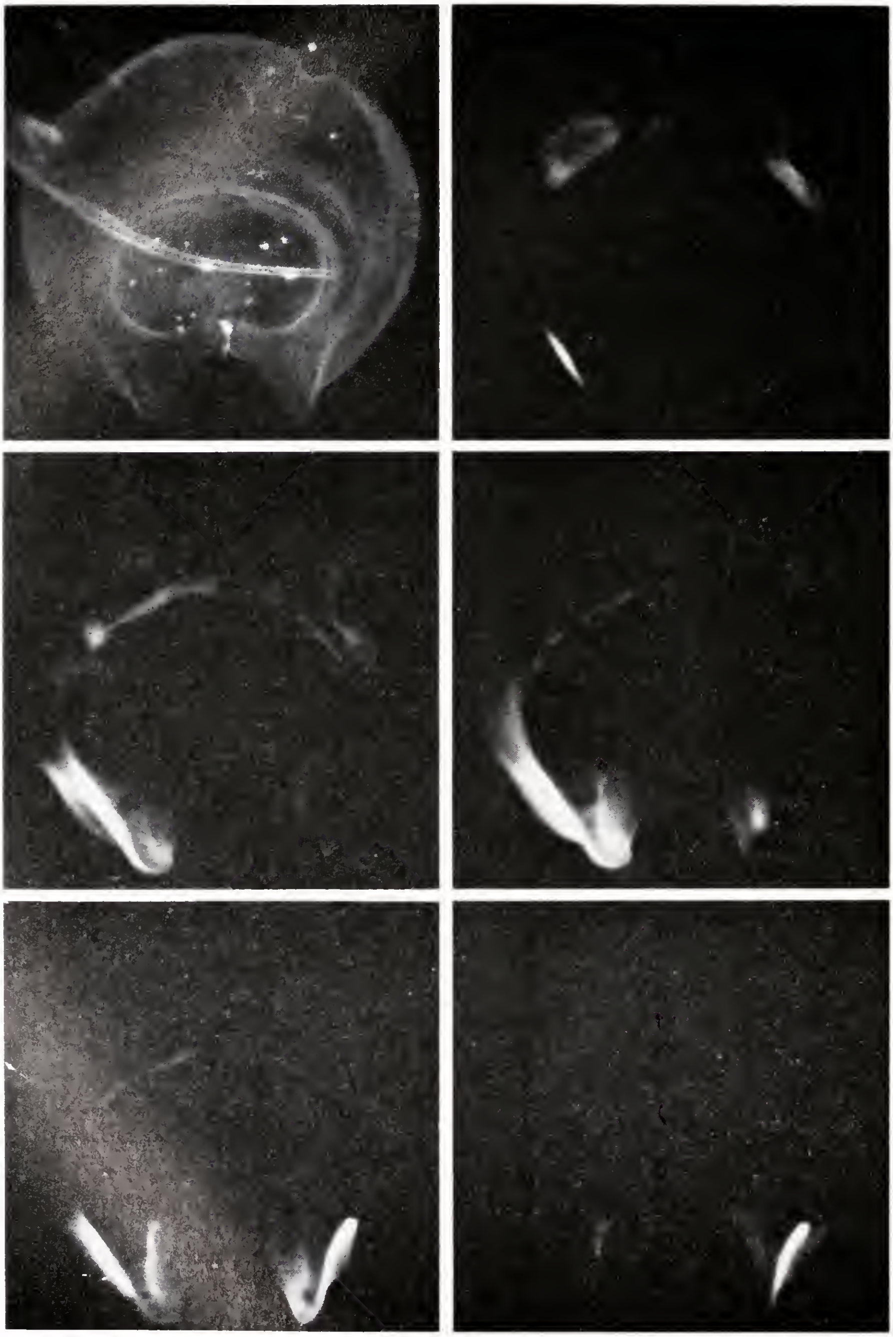

FIGURE 11. Autophotographic pictures of a nectophore during successive luminous emissions, taken through microscope and image intensifier. The first picture shows the nectophore

Deblanching occurs progressively over a period of 10 min to 1 hr according to the degree of opacity at the start. The horns are the last to clear and often remain opaque while the rest of the exumbrella has cleared completely.

Blanching and bioluminescence are evoked together by electrical stimulation. The records show that the time course of the two events may correspond closely, or one may be slightly delayed (Fig. 13). The first generalized blanching response can precede the first flash, but the minimum number of impulses needed to evoke blanching has not been ascertained. Blanching builds up to its saturation level long before the exhaustion of luminescence.

The conduction pathways

Figure 14 shows one of a number of preparations used to explore conduction routes in the nectophore. After an incision which separates the marginal ring from the exumbrellar ectoderm (operation A) impulses evoked at St1 or St2, on the ring, propagate to the radial muscles of the velum and also to those of the pseudovelum. Since the latter lie in the endoderm, and since there are no alternative routes by which impulses could enter the endoderm, it is assumed that they enter at some point around the margin. Direct connections between ectoderm and endoderm occur in this location in another siphonophore, *Nanomia* (C. L. Singla, personal communication).

Stimulation on the exumbrellar epithelium at St3 fails to excite the velar muscle but the pseudovelar fibers contract. This suggests that impulses evoked in the exumbrellar ectoderm can pass around to the back of the nectrophore, enter the axial endoderm canal and travel through it to the subumbrellar side. They do not then pass out into the ectoderm again, showing that conduction between the two layers in this region is polarized inward.

Confirmation that the axial endoderm canal is the conduction route in the previous experiment comes from adding the destruction of the axial canal (operation B) to a nectophore already bearing the incision A. There is no change in the response following stimulation at St1, but stimulation at St3 now fails to excite the endodermal radial muscles.

Stimulation on the exumbrellar surface at S3 in an intact, unoperated nectophore evokes contraction first of the pseudovelar muscle, then, after a long delay, that of the velum (Figs. 7, 8). Presumably impulses have had to pass out via the axial canal to the back and then around to the front again via the ectoderm. After operation A or B this pathway is blocked, and only the pseudovelum responds (Fig. 15).

These experiments point unequivocally to the existence of conduction bridges between the ectoderm and the endoderm at two points, one at the margin and one

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in dim artificial light; the suction electrode used for electrical stimulation ends in the pseudovelum region. The following pictures (from left to right and high to low), integrate about ten flashes each, provoked by repetitive stimulation, as in Figure 9. The successive zones of luminescent activity are clearly shown, the strongest responses being given in the horns and at the base of the prongs. Activity can switch from one region to another. Nectophore diameter is approximately 1.5 cm.

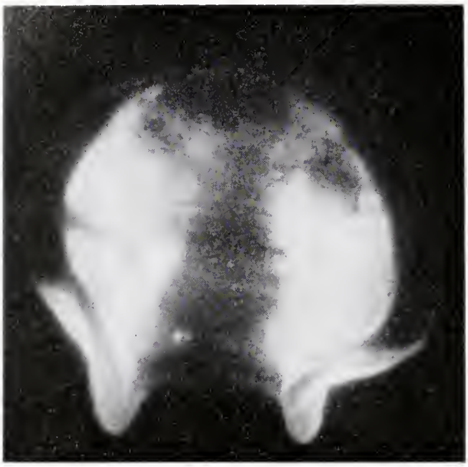

FIGURE 12. Autophotographic pictures of another nectophore during a paroxysmic response. Luminescence spreads from the horns to the back of the nectophore in successive pulsating waves, thus revealing most of the exumbrellar surface in this picture exposed several seconds. Nectophore diameter is approximately 1.5 cm.

at the back of the nectophore. These bridges have not yet been located histologically in *Hippopodius*, but conducting bridges of the sort proposed occur in hydromedusae at one of these locations, the margin (Mackie and Passano, 1968).

It follows from these observations that in the normal, intact nectophore, stimulation of either the exumbrella or subumbrella will give rise to impulses

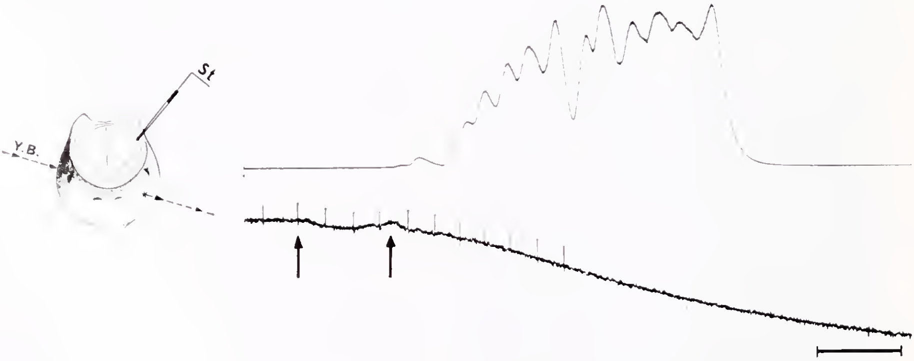

FIGURE 13. Correlation of involution, luminescence and blanching. A series of electrical shocks, shown superimposed on the blanching record (lower line, opacification downwards) evoked a multiple luminescent response (upper record, relative intensity increasing upwards) as well as involution and blanching. Involution, which occurs first, is detected as a slight deflection (between arrows). Blanching, detected by the attenuation of the yellow beam of light (Y.B.) still progresses after the end of the luminous response. Scale is 1 sec.

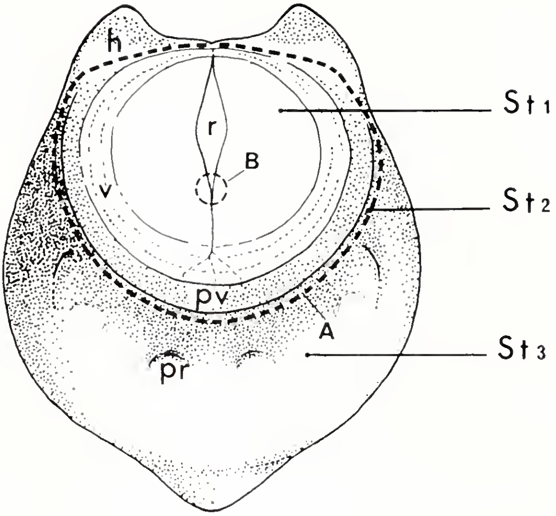

FIGURE 14. Conduction routes in the nectophore. All stimulating points (St) and cuts (dotted lines) are on the front (abaxial) side of the nectophore. Cut A is an incision through the exumbrellar ectoderm around the marginal ring separating the ectoderm within the ring, on which point St1 and St2 lie, from that outside, where St3 lies. Cut B destroys the axial canal at first point of entry into the subumbrella, separating the subumbrellar endoderm from the endoderm of the axial side. A shock at point St1 on the subumbrellar surface stimulates both the ectoderm and the endodermal lamella which lies close beneath it. This ectoderm is inexcitable, however, so the effect is simply that of exciting the endoderm. The experimental results are described in the text (h represents a horn; pr: prong; pv: pseudovelum; r: rete; v: velum).

that invade both areas. The polarization of ecto-endodermal conduction at the margin may therefore have no functional significance, since impulses have another route by which to pass to the exumbrella from the subumbrella.

Conduction between the nectophore and stem does not appear to involve direct epithelial pathways. A "translation" step intervenes, by which epithelial signals excite nervous activity in the stem tissue at its junction with the nectophore. Likewise, nervous activity in the stem appears to be translated into epithelial impulses in the nectophore. These epithelio-neural and neuro-epithelial transfer steps do not operate on a one-for-one basis, and may require facilitation. Details of these interactions will be presented elsewhere (D. Carré and G. O. Mackie, unpublished).

Morphological data

An electron microscopic survey of the conducting and contracting tissues has been carried out in order to verify previous histological accounts, and to extend them particularly with regard to junctional structure (Fig. 16).

The exumbrellar epithelium is a conventional cellular layer on the axial surface near the stem attachment, but most other areas of the epithelium are

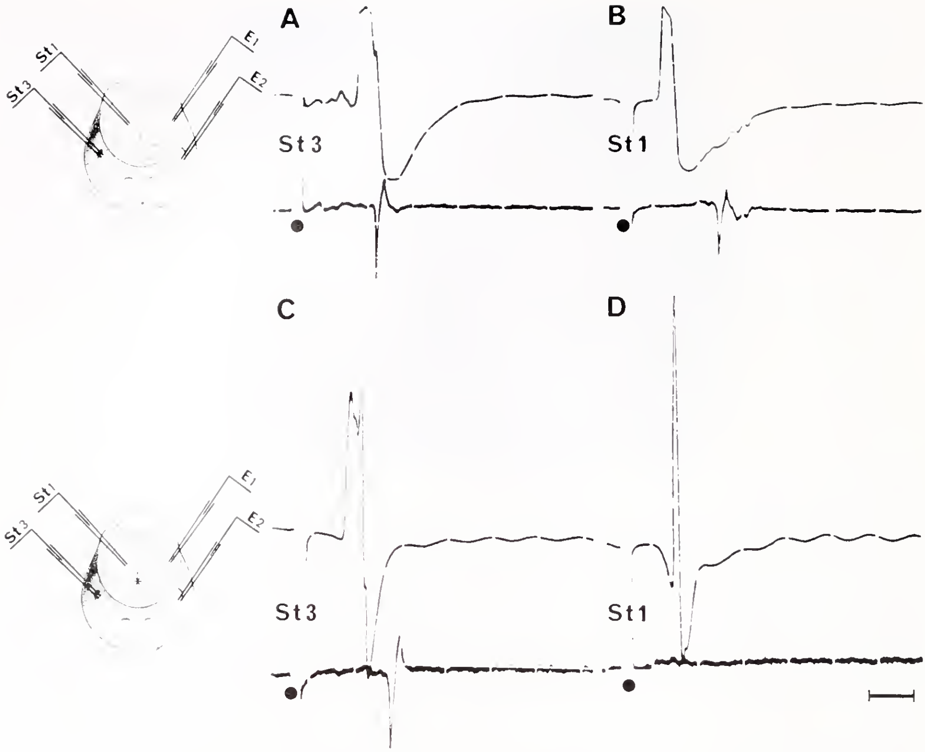

FIGURE 15. Conduction routes in the nectophore. Figures A and B are in an intact nectophore. Recording electrodes on the pseudovelum (E_1 and upper trace) and exumbrella (E_2 and lower trace) show contraction of the endodermal radial muscles and epithelial impulses, respectively. Stimuli were given on the exumbrella at St3 (Fig. A) or subumbrella at St1 (Fig. B). After destruction of the canal (operation B) excitation can still spread from ectoderm to endoderm (Fig. C), but not in the reverse direction (Fig. D). Scale is 20 msec.

syncytial (Iwantzoff, 1928; Mackie, 1965). Between cellular and syncytial zones a broader area consisting of multinucleate cells occurs. In the cellular regions, the adjacent membranes are connected by septate and gap junctions, resembling those of other hydrozoans, *e.g., Hydra* (Hand and Gobel, 1972) in their appearance and location (Figs. 11a, b). Physiologically, the whole epithelium acts as a syncytium and the membrane barriers offer no impediment to impulse spread. Gap junctions may be presumed to act as pathways for intercellular coupling (Gilula, 1973; Sheridan, 1973), while septate junctions probably act as diffusion barriers like tight junctions in vertebrates (Staehelin, 1974).

The exumbrellar cells contain no structures obviously associated with luminescence. Secretion grains occur (Fig. 11a), but similar grains occur in non luminous siphonophores (Mackie, 1965; Carré, 1975). The mesogloeal layer adjacent to the exumbrellar epithelium contains the blanching granules described previously (Mackie and Mackie, 1967) and is separated from the epithelium by a basement membrane (Fig. 16a) which is more prominent than the one found in equivalent locations in non-blanching siphonophores, and might therefore be an important structure in the mediation of this response.

Electron microscopy of the subumbrella confirms the existence of two layers of muscles, the circular striated fibers of the ectoderm and the radial, smooth fibers of the endoderm (Fig. 16c). Gap junctions connect the cells in both layers (Fig. 16d, e). Nerves have not been seen in any part of the exumbrellar epithelium nor in the muscle layers.

Discussion

The exumbrellar epithelium of the nectophore of *Hippopodius* is an anatomically well-defined layer having a large surface area and some unusual properties. Functionally, the epithelium is able to transduce external stimuli, such as mechanical or electrical shocks, into action potentials, and to propagate them over the whole surface in an unpolarized and non-decremental way. These signals induce the contraction of the radial muscle fibers localized at the margin, causing involution and cessation of rhythmic swimming. Upon spreading to the endoderm they can induce secretion by the glandular cells of the rete. They can induce, within the epithelium itself, a fast bioluminescent response, often repeated rhythmically. Finally, they can induce a long lasting blanching response in the mesoglea adjacent to the epithelium.

In its aspects of sensory transduction and conduction leading to muscle contraction, the exumbrellar epithelium of *Hippopodius* behaves like several other known examples of conducting epithelia, particularly in medusae (Mackie and Passano, 1968). The large area of the exumbrellar sheet, much of it lying at a considerable distance from the nervous ring, makes these functions easy to demonstrate. As shown by the experiments summarized in Figures 14 and 15, impulses reach the velum radial fibers directly, and can cross to the endoderm at the margin by some route which has not yet been determined. They cannot, however, cross in the reverse direction. In the normal animal, ectodermal impulses will always lead to excitation of the endoderm, since they can enter the endoderm either at the margin or at the back of the nectophore, passing into the axial canal. The two-stage involution is a direct response to the epithelial pulses, which also block endogenous swimming for an extended period. The same pulses cause secretion in the rete gland (Mackie, 1976). The other effector responses, bioluminescence and blanching, call for more extensive comment.

Bioluminescence in *Hippopodius* occurs as a surface phenomenon in a wide, flat epithelium which is also the conducting pathway for the response. The preparation should, therefore, be useful for the correlation of the temporal and spatial aspects of the bioluminescent response.

The basic photometric manifestation is a flash. Rhythmic series of flashes are characterized by facilitation, followed by fatigue. And this rhythmic flashing, often summating and ending in an "explosive" response, can be autonomous. These features are known in several bioluminescent systems, such as, for example, the octocorallians *Renilla* (Buck, 1973) and *Veretillum* (Bilbaut, 1975a, b) the hydrozoan *Obelia* (Morin and Cooke 1971a, b) or the scale worm *Acholoe* (Bilbaut and Bassot, 1977). They lead to the distinction of several problems of

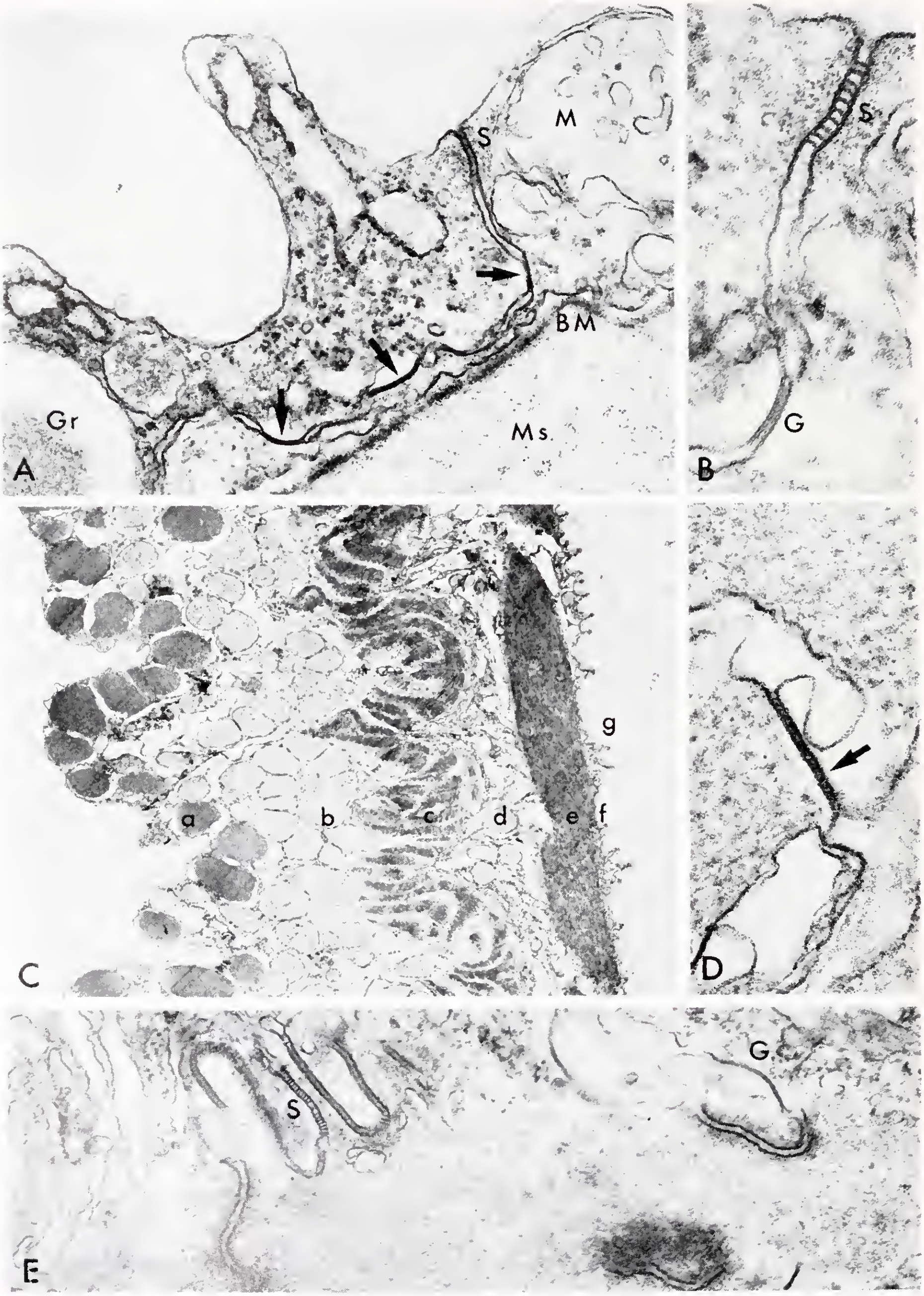

FIGURE 16. *A.* Junctional complex between two ectoderm cells of the axial cellular region of the exumbrella. The complex comprises a septate junction (S) close to the epithelial surface and several gap junctions (arrows) in the deep parts of junctional infoldings. Other abbreviations are: BM = basement membrane; Gr = secretion granule; M =

control: the excitation-bioluminescence coupling, the autoexcitation process, and the recruitment of new units of activity.

In *Hippopodius* the flash is always triggered by an epithelial potential; but the flash itself is not an electrogenic phenomenon, at least in our recording conditions: flash-inducing potentials remain the same regardless of the amplitude and the duration of the response. Since three successive pulses are needed to provoke a first flash, one may presume that the first two "prime" the mechanism of reactivity. In *Obelia* (Morin and Cooke, 1971a, b) an electrical potential is also associated with each flash, except the first one. If the initial stimulation is strong enough, autonomous rhythmic flashing occurs. It seems to represent a pacemaker like after-discharge. This manifestation recalls the "frenzy" autoexcitation state of *Renilla* during which luminous waves are propagated over the radial surface for up to an hour (Buck, 1973). However, in this case the polyps are linked by a nerve net which is assumed to initiate their luminous responses. The nerve free epithelium of *Hippopodius* provides a clear example of repetitive firing, a characteristic known in several epithelial conducting systems (Mackie and Passano, 1968).

Regarding localization, *Hippopodius* is not the first coelenterate examined with image intensification (Reynolds, 1972; Freeman and Reynolds, 1973; Buck, 1973; Morin and Reynolds, 1974) but, with its large emitting surface, it proves to be particularly interesting. Observations during flashes have shown three significant facts: first, the exumbrellar surface luminesces only in restricted area; secondly, within an active area, luminescence spreads like a wave during the flash; and thirdly, from one response to the next, the active zone changes its boundaries or even jumps from one place to another. It is thus clear that the temporal parameters of the flashes are determined by spatial features of the emitting surface. One can assume that the shape of the first responses, which originate in a rather small area within which they are nearly synchronous, comes close to illustrating the fundamental kinetics of the bioluminescent reaction. Successive flashes will increase in amplitude because of a wider surface of emission, and in duration because of the longer propagation distances involved. Double or multiple flashes presumably occur when several active zones, more or less separate or overlapping, are brought into action. The decrease in intensity at the end of the emission would be due partly to diminishing photogenic reserves, partly to a topographical retraction of the active zone which, step by step, returns to its initial boundaries, and partly to a decline in the frequency of the conducting impulses.

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mitochondrion; Ms = mesogloea. *B.* Details of sepate (S) and gap (G) junctions. *C.* Radial section through the muscular tissues lining the pseudovelum. The secretion shows, from left to right: a) secretory granules at the surface of the ectoderm, b) areas of massed mitochondria in the ectodermal cells; c) striated muscle fibers lying at the base of the ectodermal layer, d) clusters of mitochondria in the endodermal layer, e) radial smooth muscle fibers of the endoderm, f) basement membrane, g) mesogloea. In this section non-nucleus is seen either in the ectodermal or in the endodermal cells. The mesogloea separating the two epithelia is a very thin layer next to the striated muscle fibers. *D.* Gap junction (arrow) between two ectodermal striated muscle fibers. *E.* Long and sinuous junctional complex between the ends of two endodermal smooth muscle fibers. Septate junctions (S) are more conspicuous than gap junctions (G).

The fundamentally progressive facilitation of the series of flashes requires a simple mechanism to account for the extension of the active zone. The active zone and the nonresponsive zone both receive the same epithelial pulses which spread all over the epithelium. One has thus to consider that the potentially photogenic units have to be "primed" before being reactive. This priming could be due to the repetition of the epithelial pulse, as a continuation of the phenomenon observed with the first flash occurring with the third epithelial pulse. It could be due to the spread of a modification resulting from the luminous reaction itself, such as a sequestration of calcium. As established on giant cells of dipteran salivary glands (Loewenstein, 1966; Rose and Loewenstein, 1976), the intracellular free Ca^{++} concentration regulates the opening of gap junctions. The priming mechanism could also relate to the existence of different territories in the exumbrellar epithelium. The occurrence of syncytial areas (Mackie, 1965) and of different thickness may be significant in this respect.

The visible spread of ignition within the active zone which occurs during a single flash is obviously different from the overall territorial extension. The ignition wave seems able to follow the boundaries of the active zone, to rebound and to circle in whorls in such a way that every part of the active zone is explored. This recalls again the spread of luminous waves in *Renilla* (Buck, 1973).

A set of closely similar observations have been made in a recent study of the sites of activity in the elytra during luminescence of scale-worms (Pavans de Ceccatty, Bassot, Blbaut and Nicolas, 1977; Bilbaut and Bassot, 1977; Bassot and Bilbaut, 1977a, b). Elytra also have a photogenic epithelium which can be considered as a two-dimensional sheet; isolated elytra emit flashes, and rhythmic series of flashes are characterized by important and progressive variations in flash intensity, duration, delay, and frequency. These variations are also due to changes within a limited active zone and to step by step displacements of the zone. Thus, a similar mechanism of modulation related to the optional properties of the effector itself seems to be involved.

A major advantage of the elytra system is the precise identification of the morphological components. Luminescence, as well as related fluorescence, originates from photosomes, which are cytoplasmic paracrystals of endoplasmic reticulum. In *Hippopodius* the units of activity remain unknown. Their identification *in vivo* as cells, cluster of cells or subcellular components would require microscopic observation at high magnification during a flash. On sections, nothing comparable to "lumisomes" (Anderson and Cormier, 1973) or luminelles (Spurlock and Cormier, 1975) has been seen; the secretory granules observed in the epithelial cells are not yet significant enough. Nevertheless, bioluminescence is indeed a property of the epithelial cells and it seems to occur independently of any secretory discharge process.

Blanching and bioluminescence occur concomitantly only because they are responses to the same stimuli, and do not otherwise have any direct relationship. Blanching requires several seconds to reach its peak and has a considerably longer time-course than flashing. It quickly reaches a saturation level, after which further stimulation is ineffective, while luminescence can be evoked repeatedly over a long series of flashes. The nature of the reaction involving, again, calcium

(Mackie and Mackie, 1967), by which the blanching granules become visible and invisible, still requires elucidation.

In conclusion, the large epithelial surface of the exumbrella appears to play an essential role in the control of all the behavioral manifestations which follow an external stimulus. Swimming is an independent system functioning spontaneously, in rhythmic bursts, after a long period free from stimulation. A mechanical or electrical shock on the exumbrella inhibits the swimming system and induces an involution of the velum and pseudovelum. Further stimulation reinforces this response and leads to dramatic changes in the appearance of the animal, normally transparent almost to the point of invisibility: blanching by daylight and bioluminescence by night make the siphonophore suddenly visible. The observation that flashing can be repetitive, and often rhythmic, implies control by a pacemaker system. But while epithelial pulses spread everywhere over the exumbrellar surface, the effector reacts only in limited areas. Displacements and configurational changes of the active sites of light emission during successive responses are at least partially responsible for the temporal variations in the wave form of the response measured photometrically. Since triggering events in the conducting system propagate on an all or nothing basis, it follows that facilitation of the luminescent response must be due primarly to an optional property of the effector itself, which allows an increase in the number of reactive units within a population of potentially photogenic units, the remainder of which are not yet primed to react to the epithelial pulse. With this mechanism, the photogenic effector would acquire the ability to produce repeated flashes from a given photogenic reserve, and to vary successive responses.

This work was supported by a NATO Grant No. 937. The participation of G. O. Mackie was made possible under the scientific exchange program between Canada and France. We gratefully acknowldge the use of the facilities of the Station Zoologique at Villefranche sur Mer.

SUMMARY

1. Four responses are spread by through-conducting excitable epithelia in the nectophores: luminescence, blanching, muscular involution, and secretion. Swimming, which is independently controlled by the nervous system, is inhibited by epithelial impulses.

2. Luminescent flashes are correlated one for one with epithelial impulses. At least three impulses must be propagated before the first flash is recorded. Flashes sum and facilitate. Pacemaker-like after-discharges may continue after stimulation has ceased. Not all regions of the epithelium luminesce equally, and the active area can shift during a single luminescent episode, although the excitatory impulses pass across all regions equally. No steady luminescent glow has been observed. Comparisons are drawn with other luminescent systems.

3. Luminescence is generated intracellularly within the exumbrellar epithelium, but blanching (opacity) is associated with formation of granules in the adjacent mesogloea. The response builds up to saturation level within 10 sec, long before

the exhaustion of luminescence. Fading is gradual, transparency returning within an hour. Some regions blanch more strongly and fade more slowly than others.

4. Involution of the margin involves contraction of radial muscle fibres in the velar ectoderm and subumbrellar endoderm. The two groups are functionally coupled, but coupling may break down with repeated stimulation. Excitation can pass from ecto- to endoderm at the margin and either way between the two layers on the axial side of the nectophore. Thus, the endodermal effectors, including the secretory epithelium, are excited concurrently with the ectodermal.

5. Impulse conduction in the excitable epithelia and myoepithelia is assumed to involve electrical coupling mediated by gap junctions, which have been found by electron microscopy in the regions concerned.

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SIZE-DEPENDENT TIMING OF METAMORPHOSIS IN MILKWEED BUGS (*ONCOPELTUS*) AND ITS LIFE HISTORY IMPLICATIONS

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The allocation of time and resources to the conflicting demands of growth and reproduction has been an important theme in evolutionary explanations for the diversity of life history patterns among living organisms (*e.g.,* Gadgil and Bossert, 1970; Schaffer, 1974). In examining the problem of how this allocation is achieved and adaptively modified under different ecological conditions, studies of organisms with discrete life stages are particularly important, since growth and reproductive phases of the life cycle are often clearly delineated. Further, an understanding of this problem requires the integration of ecological theory with developmental and physiological considerations. These points are well illustrated by Wilbur and Collins' (1973) attempt to model control of the neurohormonal processes underlying developmental plasticity in amphibians and to account for variation in amphibian life cycles. A salient feature of their model is the role of hypothetical mechanisms which allow larvae to monitor both their rate of growth and body size; information on which the decision to metamorphose is assumed to be based. Although the existence of such mechanisms in amphibians remains to be demonstrated, there is evidence to suggest that analogous models may be relevant to the study of adaptation in insect life cycles.

Mechanisms which influence development in response to changes in body size have been described in studies of the blood-sucking bug, *Rhodnius prolixus* (Wigglesworth, 1934; Van der Kloot, 1961; Steel and Harmsen, 1971). Molting occurs only after the ingestion of a blood meal, and these studies provide strong evidence that distension of the abdomen is detected by stretch receptors which initiate the molt by triggering the release of brain hormone. Further evidence of size-dependent regulation of insect development has come from experimentally manipulating growth of larvae of a holometabolous insect, *Manduca sexta.* Nijhout and Williams (1974a, b) have shown that 5th-instar larvae molt only when they attain a size corresponding to a weight of about 5 g. When this critical size is reached, the corpora allata cease to secrete juvenile hormone, initiating the endocrine events leading to the molt. The determination of which molt will lead to a pupa is also apparently made on the basis of size (Nijhout, 1975).

Here evidence is presented that molting in *Oncopeltus* nymphs is also initiated only when a critical size is attained. Viewed in an ecological context, it is suggested that size-dependent regulation of development in *Oncopeltus* represents a life history adaptation to unpredictable conditions for larval growth.

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MATERIALS AND METHODS

Experiments were conducted with nymphs of *Oncopeltus cingulifer cingulifer*, a species of particular ecological interest because of its wide Neotropical distribution (Slater, 1964; O'Rourke, 1977). Stock cultures of *O. c. cingulifer* were derived from collections made in Trinidad. Since *O. fasciatus* has been the preferred species for physiological studies (Feir, 1974), some additional experiments were conducted with *O. fasciatus* nymphs, using a stock culture derived from collections made in Missouri, to determine whether comparable results can be expected from studies of this insect. Developmental times are the same for both species despite the smaller size of *O. c. cingulifer*. Under standard laboratory conditions described below, nymphs reach adult eclosion about 21 to 25 days after hatching. Newly-eclosed female *O. c. cingulifer* adults weigh 40 to 50 mg, compared with 60 to 70 mg for *O. fasciatus* females (Blakley, 1977).

Both species had been reared in the laboratory for several generations at the time these experiments were begun. In all experiments, nymphs were reared at 27° C under a 14L:10D photoperiod. Relative humidity varied between 30 and 60%. Details of culturing technique and experimental design are given in the appropriate sections below.

RESULTS

Competence of starved nymphs to complete development

Previous investigations have shown that *O. fasciatus* nymphs starved in the late 5th (final) instar often complete development and undergo adult ecdysis (*e.g.*, Barrett and Chiang, 1967). This was also observed in preliminary experiments conducted with *O. c. cingulifer* nymphs (Blakley, unpublished data). Results also suggested that body weight is an important factor in determining whether nymphs can successfully complete development under starvation conditions. For example, a few large nymphs starved at the time they entered the 5th instar subsequently molted, although smaller nymphs starved at later stages in this instar did not.

Further experiments were conducted to examine the relationship between body weight and the ability of starved nymphs to complete development. Five days after hatching, *O. c. cingulifer* nymphs were separated and reared individually in plastic petri dishes with holes punched in the lids to allow air circulation. Each nymph was provided with about 30 to 40 milkweed seeds (*Asclepias syriaca*) and a cotton-plugged vial of distilled water for drinking. Most nymphs entered the 5th instar 15 days after hatching, and at this time (Day 15) all nymphs were weighed and subsequently starved. Because nymphs are sensitive to desiccation (Barrett and Chiang, 1967), each bug was provided with a water vial. Only female nymphs were used, since there is a sexual dimorphism for body size. The sex of the 4th- and 5th-instar nymphs is easily determined by inspection of the abdomen (Johansson, 1958).

Results of this experiment revealed that 5th-instar nymphs whose weight exceeded 21 mg invariably completed development under starvation conditions (Fig. 1). At lower weights, progressively fewer nymphs did so, although they

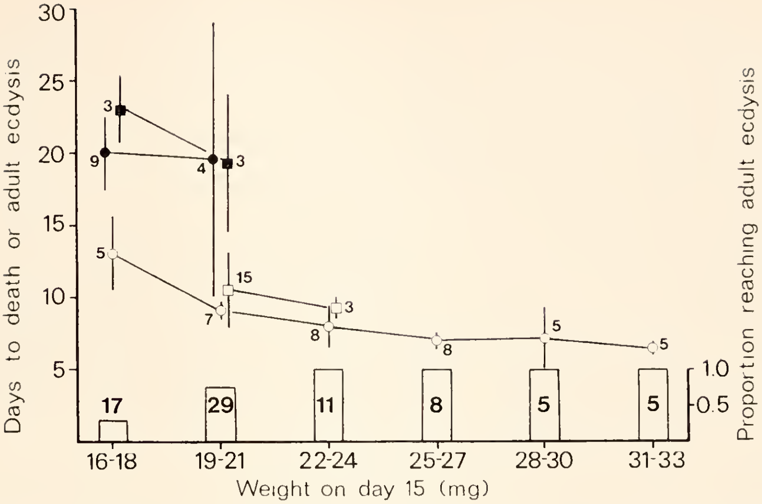

FIGURE 1. Consequences of starvation, in relation to body weight, for female 4th- and 5th-instar *O. c. cingulifer* nymphs starved 15 days after hatching. In each weight range, open symbols show the average subsequent time interval until adult ecdysis occurred. For nymphs in each weight range which did not undergo adult ecdysis, the average period for which they survived is represented by a closed symbol. Circles are averages for nymphs which were in the 5th instar on Day 15; squares are averages for nymphs which were in the 4th instar at this date. For each average, the number of nymphs represented is given and a vertical line indicates one standard deviation. Histogram shows the proportion of 4th- and 5th-instar nymphs reaching adult ecdysis in relation to body weight.

usually survived for much longer periods than was required for the completion of this instar. The importance of weight in determining whether a starved nymph would metamorphose was further emphasized by observations on nymphs which were still in the 4th instar on Day 15. Three nymphs weighing at least 22 mg subsequently molted to the 5th instar and later metamorphosed to the adult stage under starvation conditions. As with 5th-instar nymphs, the proportion of individuals completing development declined sharply at lower weights, and no 4th-instar nymphs weighing less than 19 mg reached the adult stage.

The same experiment, conducted with *O. fasciatus* nymphs, yielded comparable results (Fig. 2). As in the case of *O. c. cingulifer,* nymphs were weighed and starved on Day 15, when most nymphs entered the 5th instar. Again it was found that the proportion of nymphs which completed development declined sharply over a relatively narrow range of weights. Only 8 of 114 nymphs (7%) weighing less than 25 mg did so, while all 5th-instar nymphs weighing more than 29 mg subsequently molted. Of the 4th-instar nymphs weighing 25 to 29 mg on Day 15, 25% eventually reached the adult stage, as did 16% of the 49 nymphs in this instar weighing 20 to 24 mg. Observations made on male nymphs in this experiment gave very similar results (Fig. 3), although the proportion of 5th-instar nymphs weighing less than 30 mg which molted was slightly higher for males (17%) than for females (9%).

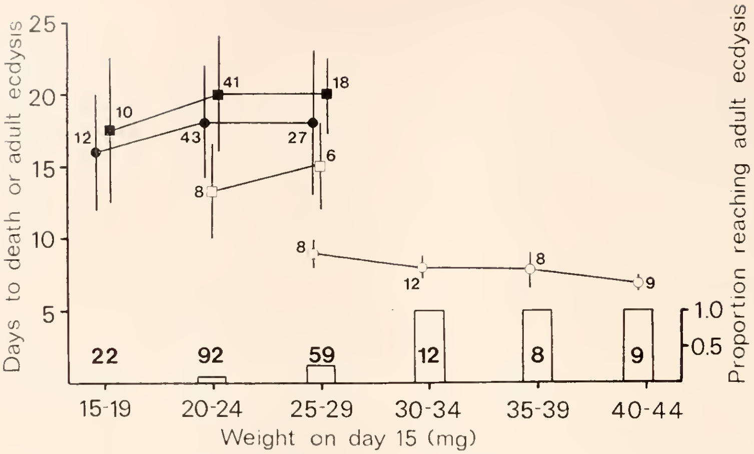

FIGURE 2. Effects of starvation from Day 15 on female *O. fasciatus* nymphs. Symbols and conventions are as in Figure 1. Note that, in contrast with *O. c. cingulifer* females, few nymphs weighing less than 30 mg completed development.

The ability of some starved 4th-instar nymphs to molt to the 5th instar and, without feeding at any time during this instar, later eclose as adults, clearly demonstrates that starvation does not invariably interrupt the normal sequence of development. It is suggested that the effect of starvation on smaller nymphs is to prevent them from reaching a critical size, at which they become competent to undergo adult ecdysis subsequently. Results for *O. c. cingulifer* females (Fig.

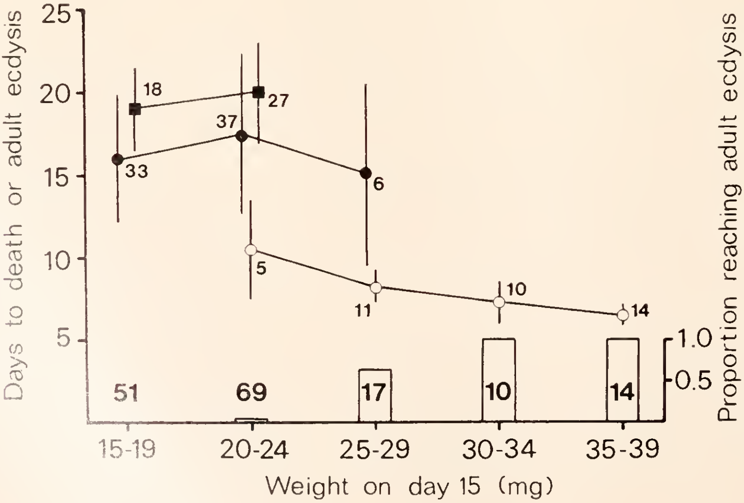

FIGURE 3. Effects of starvation from Day 15 on male *O. fasciatus* nymphs. Symbols and conventions are as in Figure 1. Note the larger proportion of nymphs in the 25 to 29 mg range which completed development in comparison with females (Fig. 2).

1), for example, are thus interpreted as follows. Nymphs entering the 5th instar at a weight of less than 19 mg which failed to metamorphose had not attained a critical size, so that without further growth they were not competent to complete development. Even a small increase in size due to the ingestion of water might allow some small nymphs to attain this size after Day 15; this may explain why five nymphs weighing about 18 mg did metamorphose. A critical size had been attained by nymphs weighing at least 22 mg and these nymphs were competent to complete development even under starvation conditions. Evidently the critical size corresponds to a weight of about 19 to 21 mg. Note that 21 4th-instar nymphs had attained this weight by Day 15 and that all but three subsequently completed development. Nymphs which attain the critical weight for metamorphosis while still in the 4th instar would, on entering the 5th instar, be competent to undergo adult ecdysis subsequently without further growth. If the critical size is taken as the minimum weight range in which at least 50% of the nymphs completed development, the values for *O. fasciatus* females (Fig. 2) and males (Fig. 3) are 30 to 34 and 25 to 29 mg, respectively. These differences are not surprising, in view of the fact that *O. fasciatus* is a larger bug, and that female *Oncopeltus* are larger than males.

Experimental prolongation of the 5th instar

To demonstrate that the timing of adult ecdysis is determined by the attainment of a critical size, further experiments were conducted with *O. c. cingulifer*

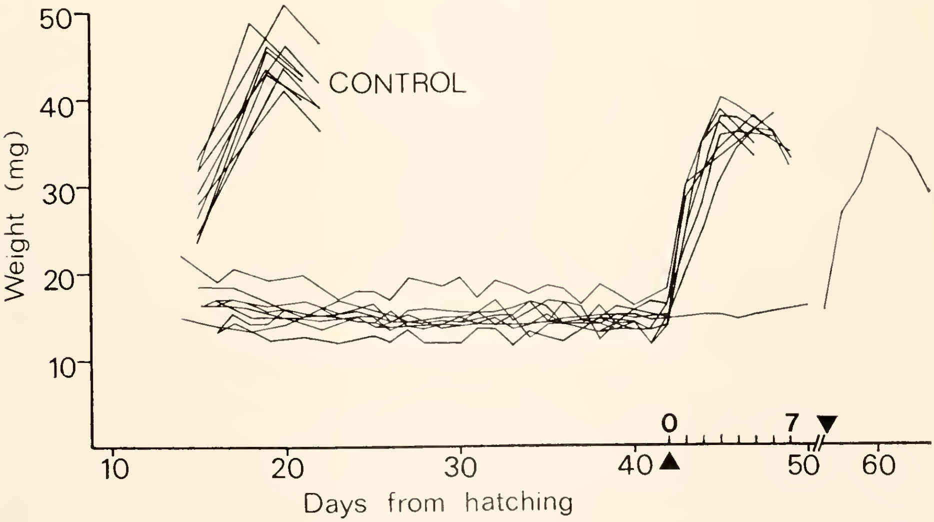

FIGURE 4. Experimental prolongation of the 5th instar of female *O. c. cingulifer* nymphs by maintaining weight below 20 mg. Each line represents a growth curve for one nymph, beginning on the day it entered the 5th instar. The final weight shown in each growth curve is that of the newly-eclosed adult. Triangles on the abcissa indicate the time at which nymphs resumed continuous feeding. Control group was allowed to feed normally throughout the 4th and 5th instars.

nymphs. Initially nymphs of both sexes were reared as in the previous experiments. Milkweed seeds were removed when a nymph's weight exceeded about 10 mg, although drinking water was supplied. All nymphs subsequently molted to the 5th instar. Nymphs of both sexes were then maintained at a weight of about 15 to 18 mg by allowing them to feed for only 10 to 30 min daily. Nymphs reaching the upper limit of this weight range were starved until their weight dropped.

Adult ecdysis was prevented in the case of female nymphs, provided they did not exceed a weight of about 20 mg (Fig. 4). To demonstrate that they were capable of molting if allowed to exceed this weight, each nymph was provided with 20 to 30 milkweed seeds on Day 42. Following the resumption of growth, all nymphs metamorphosed within 5 to 7 days (Fig. 4). The relatively small sample size reflects the fact that few nymphs survived for this length of time. Of 70 females used, 55 died before Day 42. In addition, six nymphs metamorphosed before Day 42, after their weight was inadvertently allowed to exceed 20 mg. Evidently, metamorphosis is delayed indefinitely if nymphs do not attain a critical size. In an earlier experiment, one nymph was maintained below this size until day 57 (Fig. 4).

In 14 cases, male 5th-instar nymphs were also successfully maintained below 20 mg, yet all but three molted before Day 42; adult ecdysis ensued if they attained a weight of at least 17.5 mg. This result is consistent with the lower value for the critical weight suggested for *O. fasciatus* males relative to females.

Timing of ecdysis in plant-fed bugs

Ralph (1976) and Root and Chaplin (1976) have shown that *O. fasciatus* and *O. c. cingulifer* nymphs reared on milkweed plants, rather than seeds, exhibit slow growth and development. Prolonged development under these conditions may reflect the additional time required for slow-growing nymphs to reach a critical size for molting. This possibility was investigated by observing the growth and development of individual nymphs on flowering milkweed plants (*Asclepias curassavica*) at the same temperature and photoperiod as in previous experiments. Female *O. c. cingulifer* nymphs were reared on milkweed seeds and transferred to milkweed plants 2 to 4 days after entering the 4th instar (typically lasting 4 to 5 days). Each nymph was subsequently weighed daily until adult ecdysis occurred. On entering the 5th instar, some nymphs were again provided with seeds, while the majority continued feeding on plants.

Results of these experiments are consistent with the hypothesis that metamorphosis occurs only after a critical size is attained. From previous experiments (Figs. 1, 4), this size is expected to correspond to a weight of about 20 mg in the case of female *O. c. cingulifer* nymphs. Growth curves of 5th-instar nymphs from the present experiments are illustrated in Figure 5; complete results are summarized in Figure 6. Nymphs which entered the 5th instar at a weight of at least 20 mg metamorphosed 6 to 8 days later, regardless of whether they fed on seeds or plants during this instar. Since this was also usually true of starved nymphs (Fig. 1), these results were as expected. Moreover, nymphs which entered the 5th instar at a weight of less than 20 mg, and which were then

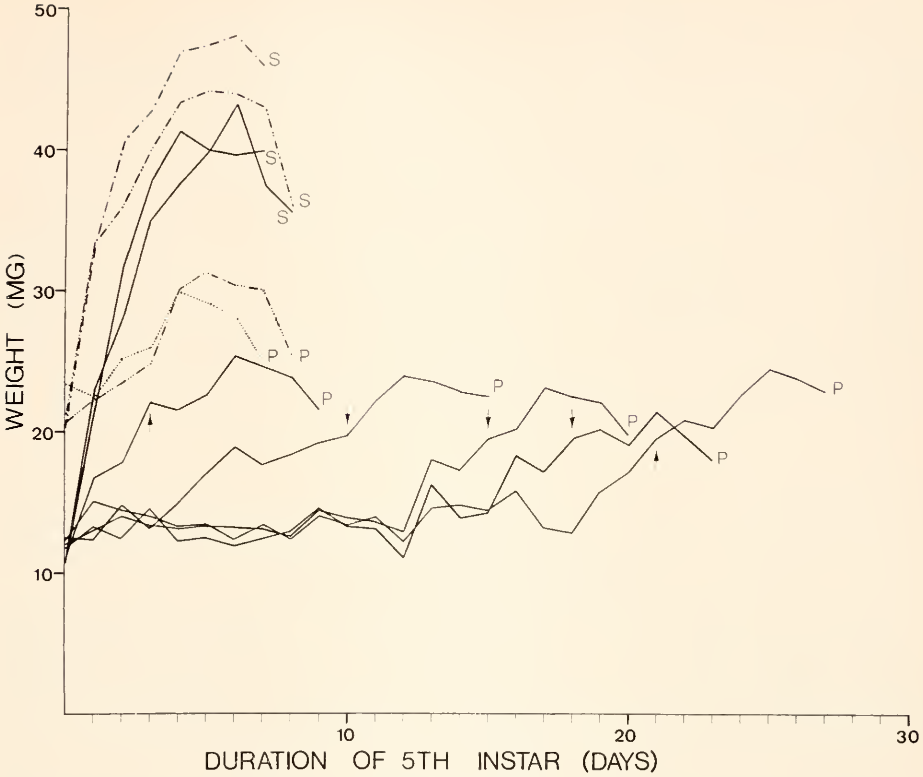

FIGURE 5. Growth curves for individual 5th-instar nymphs which fed in this instar either on milkweed seeds (S) or milkweed plants (P), showing the temporal relationship between the attainment of a critical weight of about 20 mg and subsequent adult ecdysis (the latter indicated by the final weight in each curve, which is that of the newly-eclosed adult). Nymphs which exceeded 20 mg at the outset of the 5th instar (stippled curves) metamorphosed 6 to 8 days later. Differences in diet had no effect on this time interval. Nymphs which weighed less than 20 mg at the outset of the 5th instar (solid curves) were able to attain this weight within 24 hr if fed seeds, and these nymphs also metamorphosed within a similar time interval. A longer and variable length of time was required to attain a weight of 19.5 to 20.0 mg if they were fed instead on plants. However, once this weight was attained (arrows), adult ecdysis ensued within 5 to 7 days. See Figure 6 for complete results of these experiments.

provided with seeds, attained this weight within 24 hr and also metamorphosed 6 to 8 days later (Figs. 5, 6). Note also that nymphs reared on seeds throughout their development which exceed 20 mg on entering the 5th instar typically metamorphose 6 to 7 days later (Fig. 4). Thus, under a variety of experimental conditions, nymphs weighing at least 20 mg at the outset of the 5th instar subsequently metamorphose within a relatively narrow period of 6 to 8 days.

In contrast, the duration of the 5th instar was highly variable in the case of nymphs which entered this instar at less than 20 mg and which fed only on plants.

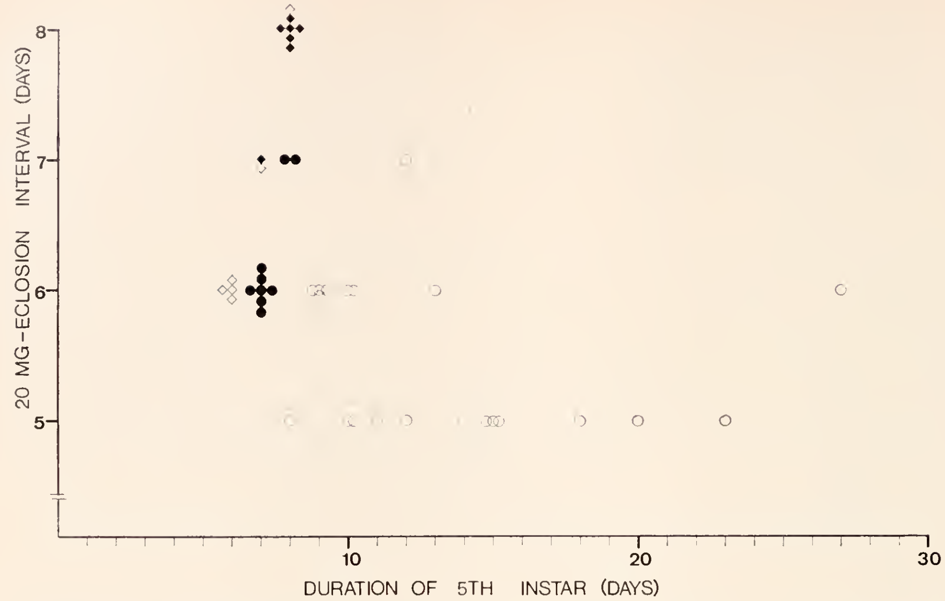

FIGURE 6. Complete results of experiments described in Figure 5. Timing of adult ecdysis is plotted in relation to two events, the time at which nymphs entered the 5th instar (abcissa) and the time at which they attained a weight of 19.5 to 20.0 mg (ordinate). *Diamonds* represent nymphs which weighed more than 20 mg at the outset of the 5th instar; the two time intervals are thus equivalent here. These nymphs metamorphosed after 6 to 8 days, whether reared on seeds (closed diamonds) or plants (open diamonds). *Circles* represent nymphs which weighed less than 20 mg at the outset of the 5th instar. These nymphs attained this weight within 24 hr and metamorphosed 6 to 7 days later when fed seeds (closed circles). When fed plants instead, the duration of the 5th instar varied considerably (8 to 27 days; open circles). However, in relation to the time at which a weight of 19.5 to 20.0 mg was attained, the timing of metamorphosis was relatively invariant (5 to 7 days later). Variation in the duration of the 5th instar is thus largely a reflection of individual differences in the time required to attain a weight of about 20 mg.

However, this varation largely reflects individual differences in the time required to attain a weight of 19.5 to 20.0 mg; all nymphs metamorphosed 5 to 7 days after reaching this weight (Fig. 6). This same interval of 5 to 7 days between the time this weight was attained and subsequent metamorphosis was also observed in the case of partially starved nymphs which resumed growth on Day 42 (Fig. 4). The reason for the slight difference between this 5 to 7 day interval and the 6 to 8 day interval observed in the case of nymphs exceeding 20 mg at the outset of the 5th instar is not known.

These results demonstrate that the existence of a critical size for molting provides an explanation for the prolonged development of slow-growing nymphs feeding on plant tissues. However, observations made on nymphs reared on milkweed plants from hatching, under the same environmental conditions as before, reveal greater flexibility in development than indicated in previous experiments. Although there was considerable variation in the duration of the 5th

TABLE I

Comparison of size, growth and developmental times for female 5th-instar nymphs reared from the 4th instar on plants or reared from hatching on plants.

Treatment	N	Pronotal width* (mm)	Duration of 5th instar (days)	Weight** (mg)		
				Day E-5	Day E-6	Day E-7
Transferred to plants in 4th instar	21	$\bar{x}$ 2.1 Range 2.0–2.2	13.3 8–27	21.4 19.5–25.0	19.5 16.5–25.8	17.4 14.4–22.3
Reared from hatching on plants	13	$\bar{x}$ 1.8 Range 1.7–1.9	11.4 7–14	15.6 12.4–21.8	13.7 11.7–18.5	13.0 9.1–18.0

* Measured on newly-molted 5th-instar nymphs using dissecting microscope with eyepiece graticule.

** Weight of 5th-instar nymphs at 5, 6, and 7 days before adult eclosion.

instar, with one exception these nymphs did not attain a weight of about 20 mg or more at 5, 6, or 7 days prior to adult eclosion (Table I). The discrepancy between these results and those obtained using nymphs transferred to plants in the 4th instar may be a reflection of the morphological dissimilarity between the 5th instar nymphs produced in the two experiments. This dissimilarity is clearly seen, for example, in measurements of pronotal width (Table I) and in other morphological features, such as antennal length, whose dimensions do not change with growth during the 5th instar. If the critical size is determined by nymphs in relation to some fixed morphological dimension, it will correspond to a similar weight for all nymphs only if they are morphologically similar. The markedly smaller 5th-instar nymphs obtained by rearing them from hatching on plants would then be expected to metamorphose after attaining a relative critical size which corresponds to a lower weight than 20 mg.

DISCUSSION

Results presented here are consistent with the hypothesis that 5th-instar *Oncopeltus* nymphs are able to assess their body size and that when a critical size is attained, physiological processes are initiated which lead to adult ecdysis. Although the nature of these processes has not been examined in this investigation, there is evidence from studies of 5th-instar *O. fasciatus* nymphs that stretch receptors are involved in their initiation at a critical size (F. Nijhout, Duke University, in preparation). That our experiments concern only the size-dependent timing of adult ecdysis does not imply that this phenomenon is unique to that particular molt. Preliminary results suggest that size may also play a similar role in the timing of earlier molts (Blakley, unpublished data).

Delayed molting under nutritional conditions which impose slow growth may effectively result because of the need to attain a critical size. In contrast, the critical weight of about 20 mg for adult ecdysis in female *O. c. cingulifer* nymphs, for example, may be attained by rapidly-growing nymphs even before they enter the 5th instar, as indicated by the weights of some 4th-instar nymphs in Figure 1.

Provided the critical weight is attained or exceeded at the outset of the 5th instar, the timing of metamorphosis is unaffected by further growth and invariably occurs 6 to 8 days later.

The fact that the critical weight for adult ecdysis may be attained by nymphs in the late 4th instar raises the problem of what determines the nature of a molt. If body size plays a role in determining which molt results in metamorphosis to the adult stage (*cf.* Nijhout, 1975), why do such 4th-instar nymphs not subsequently metamorphose as adults, rather than molting to the 5th instar? Since rapidly-growing nymphs do not attain a weight of 20 mg until 3 to 4 days after entering the 4th instar, which has a duration of 4 to 5 days (Blakley, unpublished data), it is suggested that the nature of the following molt has already been determined by the late 4th instar, and is unaffected by subsequent changes in size (or weight). Although it would be of interest to observe the effect on the following molt if a nymph attained a weight of 20 mg at the outset of the 4th instar, it seems unlikely that nymphs could ever attain this size so early in their development.

Milkweed bugs in the *Erythrischius* subgenus (including *O. fasciatus* and *O. cingulifer*) have a Neotropical distribution, with the exception of some migrant populations of *O. fasciatus* (O'Rourke, 1977). Tropical host milkweeds of these insects, notably *A. curassavica,* are an unreliable source of seeds because of their asynchronous phenology (Root and Chaplin, 1976; Blakley, 1977). As a consequence, nymphs are confronted with the possibility at any time in their development that seeds may be unavailable on a host plant, forcing them to feed on other plant structures (Blakley, in preparation). These considerations suggest that development of nymphs under natural conditions is influenced by factors affecting growth which may vary unpredictably.

In this context, size-dependent initiation of molting provides a means of adjusting the timing of life history events to prevailing ecological conditions. Since the reproductive success of adults is positively correlated with body size at eclosion (Blakley, 1977), it may be advantageous for small nymphs to delay molting and thus obtain additional time for further growth. Evidence from studies of 5th-instar nymphs previously reared on plants for different lengths of time (Table I) suggests that long delays in development of nymphs which do not have access to seeds in early instars may be avoided by adjustments in the size below which molting is delayed. If this interpretation is correct, further studies of the relationship between growth and subsequent morphological size in the following instar will be required to determine how this is accomplished.

While the relationship between adult body size and reproductive success suggests an explanation for the role of body size in the timing of metamorphosis, there are also obvious disadvantages in prolonging the larval period, because of increased pre-adult mortality. Depending on the relative advantages and disadvantages, the size at which molting is initiated may be modified by natural selection. Evidence of geographic variation in body size in *O. fasciatus* and other species (Blakley, Dingle and Klausner, in preparation) suggests that such modifications have occurred in different selective environments. This issue will be examined in a future communication.

We wish to thank Barbara Stay, Hugh Dingle and Christer Solbreck for reading the manuscript and offering useful comments and suggestions. Comments by an anonymous reviwer on an earlier version of the manuscript greatly helped in clarifying our ideas. We also thank R. Bodenweiser for sharing her insights into ecological aspects of insect development. This study was supported by NSF grants GB-40822 and DEB 73-01424-AO1 to Hugh Dingle.

SUMMARY

Experimental alteration of growth of *Oncopeltus* nymphs suggests that adult ecdysis occurs only after a critical size (indexed by weight) is attained. Nymphs which exceed this size at the outset of the 5th (final) instar metamorphose 6 to 8 days later. Nymphs which attain this size later in the 5th instar subsequently metamorphose 5 to 7 days later.

Studies of *O. c. cingulifer* and *O. fasciatus* suggest that interspecific differences in adult body size provide an indication of differences in the critical size for metamorphosis. Further, sexual dimorphism in body size also indicates a dimorphism in the critical size for metamorphosis.

The ability of *Oncopeltus* nymphs to assess their size provides a means of adjusting development in response to variable conditions affecting growth. Delayed metamorphosis in slow-growing nymphs provides additional time for further growth. This effect may be advantageous, since reproductive success declines with decreasing adult body size.

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PENETRATION OF MOLLUSCAN AND NON-MOLLUSCAN MINERALS BY THE BORING GASTROPOD *UROSALPINX CINEREA*

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The predatory shell penetrating habit characteristic of the most familiar families of recent boring gastropods, the Naticidae and the Muricidae, probably arose first in the Naticacea during Upper Cretaceous times, some 100 million years ago (Carriker and Yochelson, 1968; Sohl, 1969). During this long geologic history, boring gastropods evolved a unique mechanism for penetration of the exoskeleton of shelled invertebrate prey (Carriker and Williams, 1978).

The exoskeleton of these prey, so far as is known, consists primarily of calcium carbonate in calcitic, aragonitic, or vateritic crystalline form, and, secondarily, of a variety of trace and minor elements and an organic matrix (Carriker and Williams, 1978; Dodd, 1967; Milliman, 1974; Taylor, Kennedy and Hall, 1969). In view of the antiquity of the mechanism, it is tempting to hypothesize that the mechanism is specific for calcium carbonate and that boring snails can penetrate only this substratum. That this may not be the case, however, was suggested by Carriker, Scott and Martin (1963), who obtained faint etchings when accessory boring organs of snails were applied to the polished surface of human dentin and enamel (calcium phosphate in the form of hydroxyapatite). Although probably valid, these tests were carried out with excised accessory boring organs. The tests should be repeated using accessory boring organs of live, normally boring snails.

Recent ultrastructural studies (Carriker, 1978) demonstrated a marked variation in the solubility of different portions of the mineral components of shell units when etched by the ABO secretion. The differential solubilization may have resulted from variation in the solubility of intracrystalline organic components as well as of trace and minor mineral constituents (see also Travis and Gonsalves, 1969). However, no studies have been reported correlating the differential solubility of identified shell constituents. A first step in such an investigation could test the capacity of boring snails to penetrate a variety of isolated non-molluscan minerals which may be present in the shell in trace and minor concentrations (Travis, 1968a, b).

This paper reports the results of an experimental investigation of the capacity of the muricid boring snail *Urosalpinx cinerea follyensis* Baker to penetrate several kinds of molluscan and non-molluscan minerals. The results are discussed with reference to the specificity of the boring mechanism for different substrata and differential dissolution of shell by the secretory product of the accessory boring organ.

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Materials and Methods

Animals

Actively feeding adult *Urosalpinx cinerea follyensis* ranging in height from 38 to 46 mm were employed in the study. Snails were collected in Wachapreague, Virginia, and maintained in the laboratory in rapidly flowing seawater with oysters (*Crassostrea virginica*) and mussels (*Mytilus edulis*) obtained in the vicinity of Woods Hole, Massachusetts. The laboratory study was begun in 1970 and concluded two years later. During the winter, snails were maintained in seawater warmed to room temperature and allowed to reach gas phase equilibrium in a heat exchanger (Carriker and Van Zandt, 1973). Temperature of the flowing seawater in which the tests were run ranged from 16 to 21.6° C, salinity varied from 30.4 to 32.0‰, and the pH ranged from 8.0 to 8.2.

Mineral samples

Fifteen different biogenic and abiogenic minerals were tested (Table I). Shells of *Spisula solidissima, Crassostrea virginica,* and *Anomia simplex,* used as controls, were obtained from animals in the Woods Hole region. Radulae were extracted from *Urosalpinx cinerea follyensis* collected in Wachapreague. Normal second teeth from young people, extracted for reasons of spacing, provided human enamel and dentine. Fresh beef bone (femur) was purchased at a local meat market. Nine relatively insoluble, inorganically formed minerals were ordered from Ward's Natural Science Establishment.

At least three wafers, approximately 1 mm in thickness and ranging from 5 to 8 mm in broadest dimension, were sawed from each mineral with a diamond wafering blade on a Gillings-Hamco thin sectioning machine. One surface of each wafer was then sanded and polished with alumina (particle size 0.05 m) on a metallurgical lap wheel. Fine scratches remaining on the polished surface of the test samples provided a background and contrast to solubilized surfaces.

Tests

Sample wafers were exposed to penetration by live, normally boring snails in valve models (Carriker and Van Zandt, 1972a). After a snail had bored for a day or two into the valve of a live oyster (approximately 6 cm long), the free (unbored) valve and flesh of the oyster were removed gently under seawater, and the remaining half-shell, boring-snail preparation was placed, inner surface of the valve facing up and the snail suspended underneath, over an oval-shaped opening on a small table-like support. The valve model and support were then positioned on a manipulated stage in gently running seawater under a binocular microscope, the inner surface of the oyster valve slightly above the meniscus of the seawater (see Fig. 1, Carriker, Williams and Van Zandt, 1978). As boring approached the inner surface of the valve, the borehole could be seen through the translucent calcitostracum. Just before the snail perforated the valve, the surface of the valve around the emerging borehole was rinsed with fresh water, dried, and a thin layer of hot paraffin was applied to the shell surface to prevent possible chemical interaction between the test sample, oyster shell, and seawater. The sample, polished surface in contact with the oyster valve, was then positioned directly over the emerging borehole and cemented in place with Coecal, a slowly hardening calcareous material used for making dental molds. Next, the preparation, with the snail still at the borehole, was submerged in seawater and left there for approximately 24 hr. Borehole sites were chosen on the flat valve of oysters to provide a close contact between the sample and valve surfaces.

In preliminary trials, plaster of Paris was employed to cement the test sample to the oyster valve, but proved less workable than Coecal. Snails abandoned samples which were incompletely cemented to the oyster valve, allowing seawater to enter between the sample and the valve. To insure a water-tight seal, each sample was cemented carefully to the valve surface along its entire perimeter, and an additional thick layer of Coecal was spread over the sample and adjacent valve surface (Fig. 1). Snails bored easily through both plaster of Paris and Coecal without obvious deleterious effects.

Snails enlarged the borehole from the initial break to a diameter which would fully admit the proboscis and the accessory boring organ in about six hours (Carriker and Van Zandt, 1972a, b). Figures for duration of penetration in Table I represent the approximate time that the proboscis and accessory boring organ were in full contact with the surface of the test samples. In several cases, snails abandoned the preparation during the night and it was not possible to determine the actual time of penetration.

At the termination of approximately 24 hr, each snail, if still present, was dislodged from its borehole. The sample and Coecal were removed from the oyster valve, rinsed quickly in distilled water, dried with a current of warm air, and stored in a vacuum desiccator.

Average maximum depth of incomplete boreholes in experimental samples was determined with a Wild M-20 compound microscope provided with incident illumination (epi-attachment) and a scale (each interval = 1 micron) on the fine focus knob for measuring vertical distances.

The effect of secretion of the accessory boring organ on radular teeth was tested as follows. Radulae were excised under a binocular microscope from large adult *Urosalpinx cinerea follyensis,* washed in distilled water, spread between fragments of cover glass, and allowed to dry in the sun. After drying, each radula was mounted on a strip of thin plastic cover glass by application of small drops of Duco cement at the ends and at intervals along the length of the radula. Each radula, still mounted on the plastic strip, was then placed over the open borehole in a valve model just after the proboscis had been retracted and the accessory boring organ was being extended. The preparation was removed from the borehole the instant the accessory boring organ was withdrawn at the termination of the period of chemical activity and before the proboscis was again inserted in the hole. A length of the radula not yet employed in rasping over the bending plane of the odontophore was exposed to the secretion of the

TABLE I

Penetration of molluscan and non-molluscan minerals by Urosalpinx cinerea. *pK_{sp} = negative logarithm of K_{sp}. ( ) = snail abandoned hole before the test period ended.*

Minerals			Penetration		
Source of test sample, mineral, major constituents	Hardness, Knoop values (kg/mm²)	Solubility product (as pK_{sp})	Duration (hr)	Depth (μm)	Characteristics, dissolution
Crassostrea virginica shell, calcite: $CaCO_3$ + organic matrix	244[1]	6.2[5]	18.3 17.8 18.5	230 210 180	Shallow borehole Shallow borehole Shallow borehole
Spissula solidissima shell, aragonite: $CaCO_3$ + organic matrix	—	6.1[5]	20.5 19.5 20.0	280 180 180	Shallow borehole Shallow borehole Shallow borehole
Anomia simplex shell, aragonite: $CaCO_3$ + organic matrix	—	6.1[5]	18.5 18.0 (left)	205 120 (60)	Shallow borehole Shallow borehole Shallow borehole
Urosalpinx cinerea, radula[3]: $CaCO_3$, Sr, Mg, Bo, Si, Zn, Fe + organic matrix	200[1] 407	Practically insoluble	0.6 1.6 2.6	0 0 0	No dissolution No dissolution No dissolution
Beef bone, hydroxyapatite: $Ca_{10}(PO_4)_6(OH)_2$ + organic matrix	—	54.6[7]	17.0 18.0 21.0	90 55 50	Shallow borehole Shallow borehole Shallow borehole
Human tooth, hydroxyapatite: $Ca_{10}(PO_4)_6(OH)_2$ + organic matrix—enamel —dentine	430[2] 430[2]	52.2–57.2[6]	17.5 (left) 17.5 18.0	15 (10) 25 5	Slight depression Slight depression Slight depression Slight etching
Strontianite: $SrCO_3$	149	10.0[4]	18.0 13 (left) (left)	110 (40) (10)	Shallow borehole Slight depression Slight depression
Anhydrite: $CaSO_4$	149	4.6[4]	17.0 (left) 12.5 (left) (left)	(25) (15) (10)	Slight depression Slight depression Slight depression
Witherite: $BaCO_3$	149	9.3[4]	(left) (left) (left)	(20) (5) (?)	Slight depression Slight etching Questionable
Magnesite: $MgCO_3$	149–296	5.0[4]	(8.5 (left) (left)	Trace (0) (0)	Slight etching No dissolution No dissolution
Siderite: $FeCO_3$	163–296	10.5[4]	20.0 (left) (left)	0 (0) (0)	No dissolution No dissolution No dissolution
Smithsonite: $ZnCO_3$	163–296	10.7[4]	(left) (left)	(0) (0)	No dissolution No dissolution

TABLE 1—*Continued*

Minerals			Penetration		
Source of test sample, mineral, major constituents	Hardness, Knoop values (kg/mm^2)	Solubility product (as pK_{sp})	Duration (hr)	Depth (μm)	Characteristics, dissolution
Alunite: $KAl_3(OH)_6(SO_4)_2$	149–163	Insoluble?	14.0 (left)	(0)	No dissolution
			18.5	0	No dissolution
			18.0	0	No dissolution
Fluorite: CaF_2	163	10.6[4]	16.0 (left)	(0)	No dissolution
			15.5 (left)	(0)	No dissolution
			13.0 (left)	(0)	No dissolution
Quartz: SiO_2	820		(left)	(0)	No dissolution
		Insoluble	(left)	(0)	No dissolution

[1] Carriker (1969).
[2] Trautz (1967).
[3] Carriker and Van Zandt (1972a).
[4] Kolthoff, Sandell, Meehan and Bruckenstein (1969).
[5] Berner (1976)—calcite and aragonite in seawater.
[6] Patel and Brown (1975).
[7] Brown and Chow (1976).

accessory boring organ. The upper surface of the valve was positioned just above the surface of the seawater so that the radula would be in contact only with secretion of the accessory boring organ. Three radulae were tested, one for 38 min, a second for 97 min, and the third for 154 min. These intervals were chosen because during each normal cycle of rasping and chemical activity the radula is exposed to secretion in the borehole for about one minute, and it takes a snail 150 1-minute rasping periods, or a total of 150 min of exposure to secretion of the accessory boring organ, to penetrate oyster shell 1 mm in thickness (Carriker and Van Zandt, 1972a). After exposure to the secretion for the designated interval, each radula was rinsed in distilled water, dried in a stream of warm air, and stored in a vacuum desiccator.

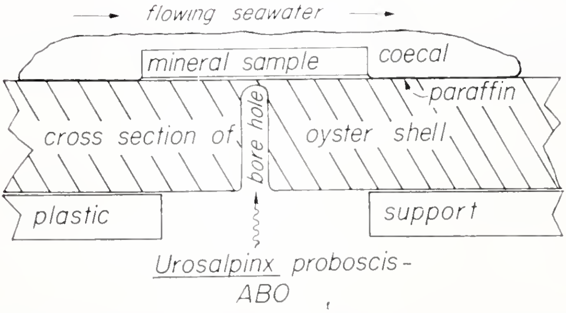
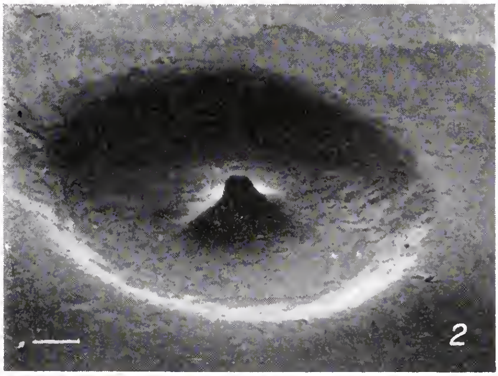
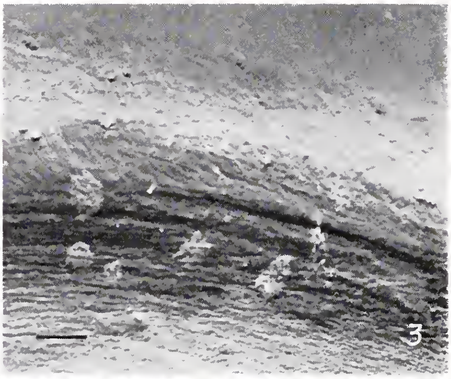
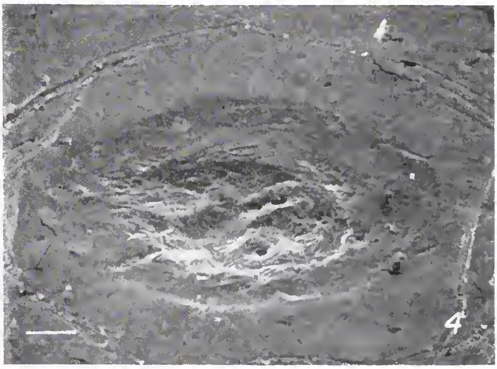
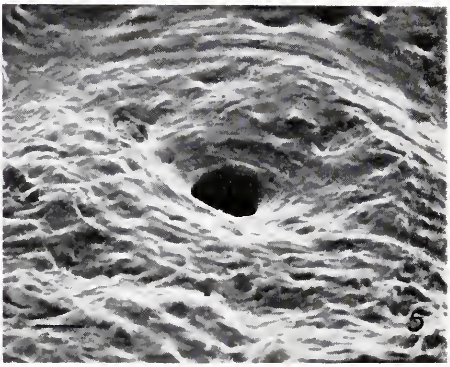


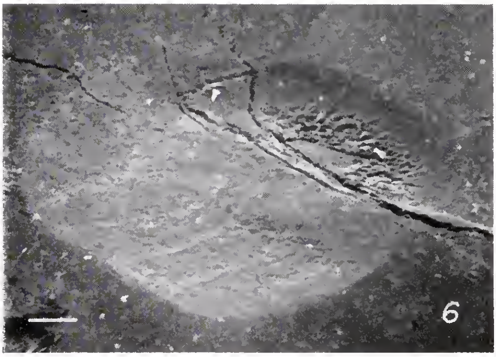
FIGURE 1. Cross sectional diagram of device used to test penetrability of mineral samples by *Urosalpinx cinerea*. Diameter of borehole 1 mm.

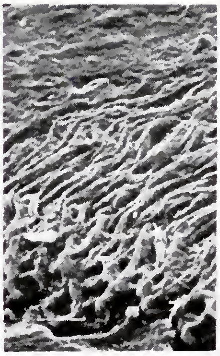


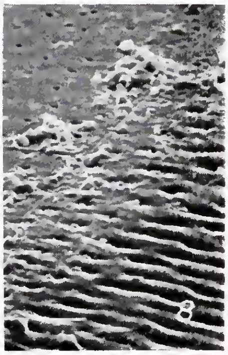












Figures 2–20 are scanning electron micrographs.

FIGURE 2. Incomplete borehole in shell of *Anomia simplex*. Scale bar equals 0.2 mm.

FIGURE 3. Portion of beveled edge of borehole in Figure 2. Scale bar equals 0.05 mm.

FIGURE 4. Shallow borehole in beef bone. Small holes in borehole are Haversian canals. Scale bar equals 0.13 mm.

FIGURE 5. Enlargement of a Haversian canal in the borehole in Figure 4. Scale bar equals 20 μm.

FIGURE 6. Shallow borehole in human tooth. Lower left portion of hole is in enamel, and upper right is in dentine. Cracks through wafer resulted from drying. Scale bar equals 0.1 mm.

One of each of the triplicate samples of each test mineral and the three radulae were examined with a scanning electron microscope (Cambridge Stereoscan Mark II) at magnifications ranging from 16 to 2500 ×. Mineral wafers were removed from the Coecal, cemented with silver paint to scanning electron microscope pin stubs 1 cm in diameter, and dried in an oven for about 48 hr before coating with carbon and gold for viewing in the scanning electron microscope. Radulae were removed from the plastic cover glass strips, transferred to double adhesive tape on stubs, and secured at the ends to the tape with silver paint prior to coating with carbon and gold.

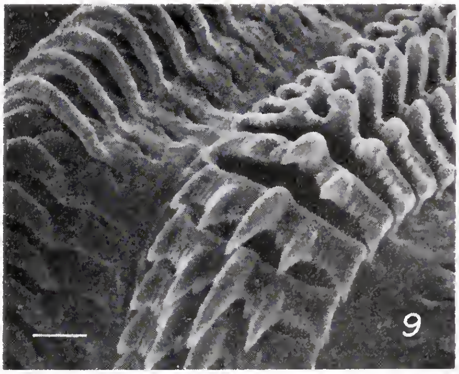
RESULTS

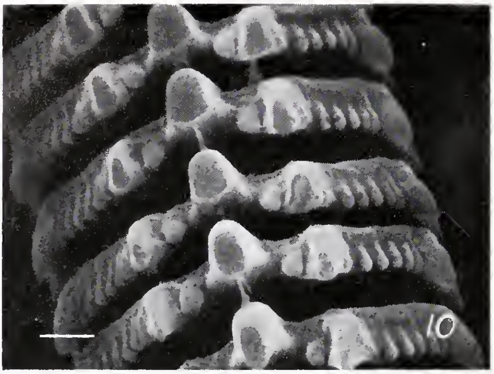
All biogenically formed calcareous structures, except radulae, exposed to normally boring *Urosalpinx cinerea* on valve models were penetrated (Table I). The rate of penetration of the mineral wafers decreased in the following order: calcite and aragonite, strontianite, bone and tooth hydroxyapatite, anhydrite, witherite, and magnesite. At least at the magnifications employed with the scanning electron microscope, the abiogenic minerals, siderite, smithsonite, alunite, fluorite, and quartz did not seem to be affected either by radular rasping or secretion from the accessory boring organ. Snails remained on calcitic wafers of *Crassostrea virginica,* aragonitic wafers of *Spisula solidissima,* and hydroxyapatitic wafers of bone for the full 24 hr of the experiment. One snail abandoned an aragonitic wafer of *Anomia simplex,* and another, a hydroxyapatitic wafer of human tooth prior to termination of the testing period. In most cases of abandonment, snails left the preparation during the night or during periods when we were absent, so it was difficult to approximate the amount of time on the wafer. Of the total of 15 tests performed on biogenically formed calcareous minerals, only two (or 13%) of the snails left the sample on the model before the experiment was terminated; whereas of the 25 tests on abiogenically formed minerals, 20 (or 80%) of the snails crawled off the sample before the end of the test.

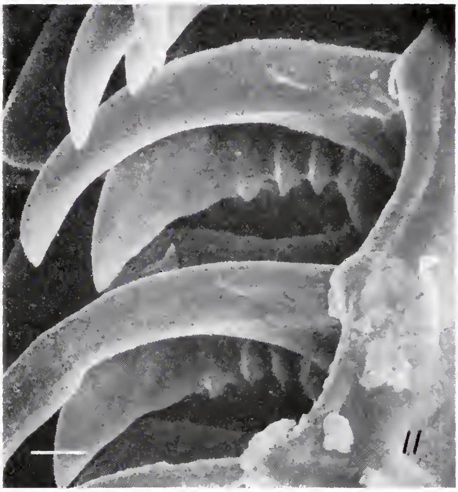
Shell of *Crassostrea virginica, Spisula solidissima* and *Anomia simplex* was bored to approximately the same average depth (Table I, Figs. 2, 3). The shape and size of incomplete boreholes were characteristic of holes made by *Urosalpinx cinerea* when penetrating normal prey shell (Carriker and Yochelson, 1968). An exception to this was the curious mount left in the center of the borehole in one of the wafers of *Anomia simplex* by one of the snails (Fig. 2). Ultrastructural examination of the fractured surface of the top of the mount showed normal nacreous lamellae; energy dispersive X-ray analysis of the top and sides of the mount disclosed no unusual concentrations of minor or trace elements (at least at the minimal sensitivity of the technique, 500 to 1000 ppm)

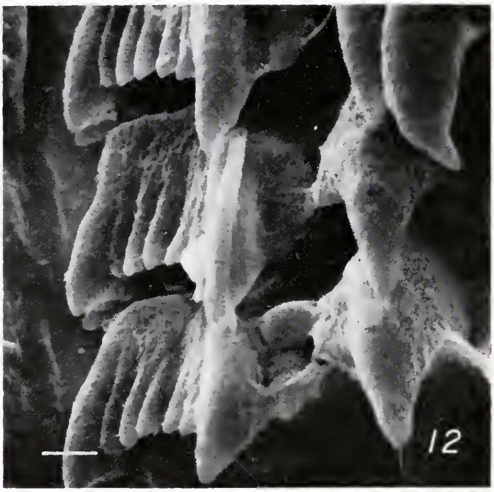
FIGURE 7. Enlargement of edge of borehole in enamel in Figure 6. Polished portion of wafer is at upper left. Partly dissolved prism ends and sides shown in lower portion of micrograph. Scale bar equals 9 μm.

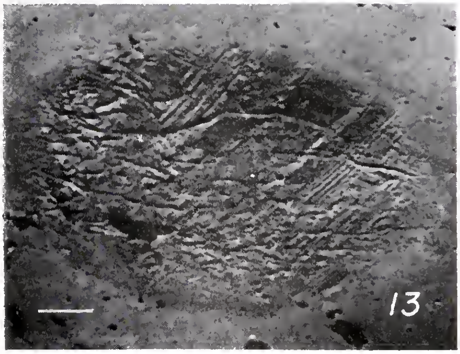
FIGURE 8. Enlargement of edge of borehole in dentine in Figure 6. Polished portion of wafer is at upper left, dissolved portion lower right. Cross sections of tubules are visible in polished portion, and oblique sections in the partly dissolved dentine. Scale bar equals 10 μm.











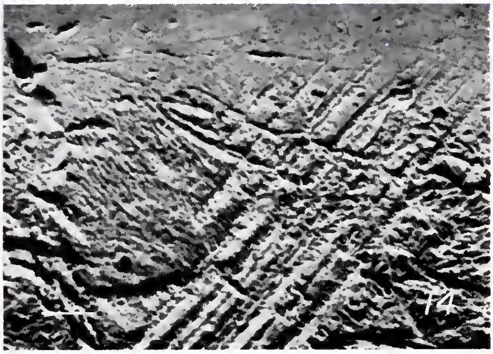


FIGURE 9. Unworn radula of *Urosalpinx cinerea* prior to use by snail for boring hole. Five-cusped teeth are in center row, and marginal teeth in row to either side. Scale bar equals 35 μm.

FIGURE 10. Portion of radula of *Urosalpinx cinerea* taken from anterior end of radula after snail bored a hole, showing extent of abrasion that takes place during shell penetration.

which might have decreased the solubility of shell in that region. Sides of the borehole were characterized by normal bevelling (Fig. 3).

Degree of penetration of beef bone (Fig. 4) was intermediate between that of shell and teeth (Fig. 6, Table I). Dissolution of bone in the borehole was uneven, though conspicuous (Fig. 4), and smoothly bevelled the rim of exposed Haversian canals (Fig. 5). The pattern of concentric rings surrounding the Haversian canal in Figure 5 suggests successive layers of mineral lamellae.

Dentine was penetrated slightly more deeply than enamel (Fig. 6, Table I). The secretion of the accessory boring organ dissolved the edges of tubules in the dentine, making them conspicuous (Fig. 8). Figure 7 suggests that the center of enamel prisms was dissolved more deeply than the perimeter.

Radular teeth of *Urosalpinx cinerea* placed on the secretion of the accessory boring organ were unaffected during exposures ranging from 0.5 to 2.5 hr (Figs. 11, 12; Table I). The wrinkled surface on the cusps of teeth in Figure 12 represents dried secretion; no blunting of cusp points or pitting of the tooth surface was evident. Compare with control radulae in Figures 9 and 10.

Of the abiogenically formed minerals, strontianite was penetrated most deeply (Figs. 13, 14). Incomplete boreholes were quite shallow in witherite (Figs. 15, 16) and in anhydrite, and less noticeable at low (Fig. 17) or higher magnifications (Fig. 18) in magnesite. Rasp marks scraped by the radula during boring were clearly evident in the relatively soft minerals strontianite (Figs. 13, 14) and witherite (Figs. 15, 16).

At magnifications employed with the scanning electron microscope, no obvious dissolution of the surface of the minerals siderite, smithsonite, alunite, and fluorite occurred (Figs. 19–22). The faint half circle in Figures 19, 20, and 22 represents residues left by the secretion at the edge of the accessory boring organ. This interpretation is supported by the persistence of original scratch lines, which are still visible without appreciable disfigurement from dissolution at the site where the accessory boring organ was applied.

The experiments demonstrated that Ca, Sr, Ba, and Mg as carbonates, and Ca both as the phosphate and sulfate, were attacked by the secretion of the accessory boring organ. On the other other hand, Fe and Zn as the carbonates, Ca as the fluoride, Si as the oxide, and K and Al as the sulfate, were unaffected.

Discussion

The capacity of *Urosalpinx cinerea* to penetrate a wide range of types of minerals potentially extends the variety of shelled prey which the snail can

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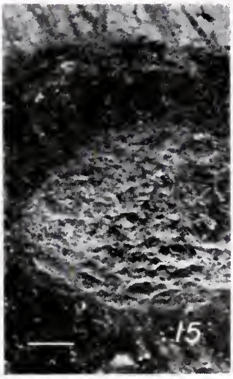
Denticles between cusps show no dissolution from exposure to secretion of accessory boring organ in borehole. Scale bar equals 15 μm.

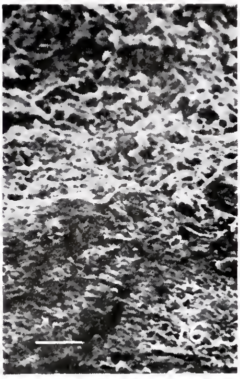
Figure 11. Teeth of *Urosalpinx cinerea* exposed experimentally to secretion of accessory boring organ for 0.5 hr. Marginal teeth in foreground and rachidian teeth in background. Scale bar equals 10 μm.

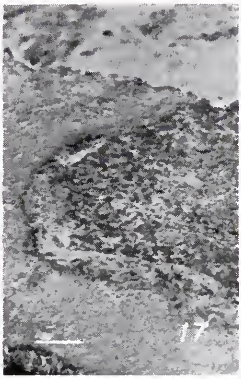
Figure 12. Rachidian teeth of *Urosalpinx cinerea* exposed experimentally to secretion of accessory boring organ for 2.5 hr. Scale bar equals 10 μm.

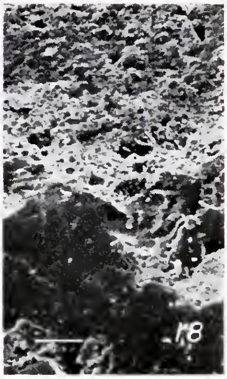
Figure 13. Shallow borehole in strontianite ($SrCO_3$). Rasp marks are evident on upper right half of borehole. Scale bar equals 0.2 mm.

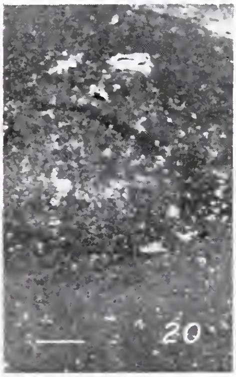
Figure 14. Enlargement of edge of borehole in Figure 13. Scale bar equals 0.05 mm.











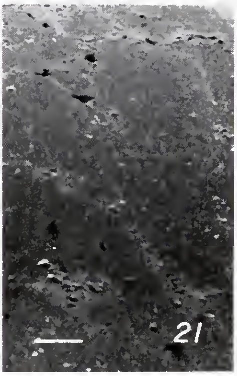


FIGURE 15. Left half of slight depression dissolved by secretion of accessory boring organ in witherite ($BaCO_3$). Rasp marks pass from etched depression onto polished surface of wafer. Scale bar equals 0.15 mm.

FIGURE 16. Enlargement of edge of depression in Figure 15. Scale bar equals 15 μm.

FIGURE 17. Left half of shallow etching made by secretion of accessory boring organ in magnesite ($MgCO_3$). No rasp marks are evident. Silver paint at upper right of micrograph. Scale bar equals 0.3 mm.

FIGURE 18. Enlargement of edge of etching in Figure 17. Scale bar equals 25 μm.

FIGURE 19. Polished surface of siderite ($FeCO_3$) to which snail applied accessory boring organ. Scale bar equals 0.2 mm.

FIGURE 20. Polished surface of smithsonite ($ZnCO_3$) to which snail applied accessory boring organ. Scale bar equals 0.25 mm.

FIGURE 21. Polished surface of alunite $KAl_3(OH)_6(SO_4)_2$ to which snail applied accessory boring organ. Scales bar equals 0.15 mm.

FIGURE 22. Polished surface of fluorite (CaF) to which snail applied accessory boring organ. Scale bar equals 0.2 mm.

penetrate. This accounts, at least in part, for the broad spectrum of species upon which it can prey: its own kind, slipper limpets, cockles, edible and ribbed mussels, soft and hard clams, scallops, oysters, barnacles, and encrusting bryozoans (Carriker, 1955; Hancock, 1959; Wood, 1968). *U. cinerea* may also feed upon small moribund crabs and the carrion of fish, and, in fact, on many other invertebrates whose flesh may be available. There are no reports on whether the snail bores through the exoskeleton of crustaceans. Boring gastropods probably have the capability to do so, as some species of Naticidae can penetrate the cases of skate eggs (Jensen, 1951), but the motility of crustaceans undoubtedly prevents this. *U. cinerea,* guided by ingestive experience and not by genetic limitation (Wood, 1968) prefers live prey (Federighi, 1931) and subsists generally on oysters, edible mussels, or barnacles as they are available (Carriker, 1955).

Calcitic and aragonitic bivalve shell wafers were penetrated at about the same rate (Table I). *Urosalpinx cinerea* also bores normal holes in the aragonitic shell of the gastropod *Murex fulvescens* (Carriker and Van Zandt, 1972a), and on valve models has excavated shallow boreholes in wafers of abiogenic calcite (Carriker, unpublished data). Because of the reported lack of attractiveness of *Anomia simplex* to *U. cinerea* (Carriker, 1955; Galtsoff, Prytherch and Engle, 1937; Pratt, 1974; Carriker and Van Zandt, unpublished), penetration of the shell wafers of *A. simplex* as readily as those of *Spisula solidissima* was unexpected. Galtsoff, *et al.,* (1937) observed that, of all the food organisms offered to *U. cinerea* in the laboratory, *A. simplex* remained untouched. They concluded that the shell of *A. simplex* affords a barrier to *U. cinerea.* Carriker (1955) in a screened enclosure in the field, found that one *A. simplex* had been partially bored and two others had been completely penetrated, but all three bivalves were still living. Microscopic examination of the completely bored bivalves showed that snails had rasped away some of the flesh but that bivalves had regenerated the mantle and secreted a thin layer of new shell over the perforations. Pratt (1974) confined *U. cinerea* with nine species of potential prey in the laboratory and all but the *A. simplex* were bored. Carriker and Van Zandt (unpublished) observed that ready-to-set larvae of *A. simplex* entered a running seawater laboratory tank in which *U. cinerea* were maintained with oysters and edible mussels, settled on the bottom of the tank and grew to a diameter of about 3.5 cm. None of the dozen *A. simplex* was bored by the 50 adult snails present, whereas oysters and mussels were consumed regularly. The present investigation demonstrated that failure of *U. cinerea* to bore valves of live *A. simplex* was not due to their inability to penetrate shell, but either to the lack of an attractive feeding kairomone, or to the presence of a feeding suppressant in the effluent of the living bivalves. Carriker's (1955) observation that living *A. simplex* were bored but not consumed, and the report by Galtsoff, *et al.,* (1937) that starved *U. cinerea* did not bore the valves of *A. simplex* but did consume the flesh of the shucked individuals, support the tentative conclusion that *U. cinerea* are suppressed by a chemical released by living *A. simplex,* and to a much reduced extent, or none at all, by fluids from shucked *A. simplex.*

Solubility of minor and trace shell minerals in the secretion of the accessory boring organ could facilitate penetration of prey shell by boring gastropods. Travis (1968a, b) found that crystalline "impurities" are present as celestite ($SrSO_4$), strontianite ($SrCO_3$), barite ($BaSO_4$), and witherite ($BaCO_3$) within the mineral cores of single prisms of the shell of *Mytilus edulis.* Two of these minerals, strontianite and witherite, were shown to be soluble in the secretion of the accessory boring organ in the present study; celestite and barite, although not tested, are probably also soluble. Insoluble, or only slightly soluble, minerals present in shell even in small amounts, could slow slightly the rate of penetration of shell, place a heavier burden than normal on the rasping phase of the shell-boring process (Carriker and Van Zandt, 1972a), and accelerate abrasion of radular cusps (Carriker, 1969; Carriker, Schaadt, and Peters, 1974). In this connection, epidemiological studies have shown that there may be an association between some trace elements, other than fluoride, and human dental caries (Legeros, Miravite, Quirolgico, and Curzon, 1977); that is, some elements, such as strontium, magnesium, and boron, are suspected of being cariostatic, while others, such as manganese, iron, lead, and copper, are suspected of being cariogenic. In the same sense the presence of some trace elements in shell of prey of boring gastropods may retard dissolution and others may accelerate it. This matter has not yet been investigated.

The decreasing rate of penetration of mineral wafers by *Urosalpinx cinerea* was shell, strontianite, bone and teeth, anhydrite, witherite, and magnesite; yet the wide range of solubility products of the minerals, tested in decreasing order, was anhydrite, magnesite, shell, witherite, strontianite, siderite, smithsonite, fluorite, bone and teeth, alunite and quartz (Table I). Thus, rate of penetration was not necessarily greater in the more soluble than in the less soluble minerals. A good example of this was magnesite, which though relatively more soluble than shell, was scarcely penetrated. Another was calcitic and aragonitic shells, which though characterized by different solubilities, were penetrated at about the same rate. The low pH (3.8–4.0) of the secretion of the accessory boring organ (Carriker and Williams, 1978) relative to the composition of the various minerals may offer an explanation. However, pH alone may not be the sole answer, as the secretion of the accessory boring organ appears to be a complex fluid (Carriker, *et al.*, 1978). There is likewise no apparent association between hardness of the mineral wafers and rate of penetration; strontianite, anhydrite, and witherite, for example, although softer than shell, were penetrated considerably less than shell. It is likely that hardness affects only the mechanical aspects of shell penetration by the radula (Carriker and Van Zandt, 1972a).

All abiogenic minerals, which lack the organic framework of molluscan shell, were penetrated slowly, if at all. This observation suggests that the organic matrix, among other possible factors, facilitates penetration of shell by boring gastropods. This suggestion is supported by the fact that in holes bored by *Urosalpinx cinerea,* especially in prismatic and myostracal regions, solubilization of organic matrix noticeably precedes dissolution of the mineral crystals (Carriker, 1978). Differential dissolution of shell is significant because initial solubilization of organic matrix facilitates removal of shell units from the surface of the borehole by the radula (Carriker, 1977), and bares a greater surface area of the calcareous components to the etching action of the secretion than would be the case on a smooth mineral surface. This point is underscored by strontianite, which was penetrated at about one-half the rate of shell. Strontianite, though softer but less soluble than shell, was also the most rapidly penetrated of all the abiogenic minerals tested. This could be explained by the fact that this mineral is mineralogically close to aragonite and could thus be more readily attacked than other abiogenic minerals. Fluorite, also softer and less soluble than shell, is not dissolved at all by the secretion of the accessory boring organ. Possibly this mineral acts as a depressant. The question might well be posed whether fluorite would be more rapidly attacked by the snail if the mineral were deposited biologically in an organic matrix.

Carriker (1978) hypothesized that differences in distribution and concentration of trace and minor minerals in the cores of shell units, and differential solubility of these minerals in the secretion of the accessory boring organ, could account in part for the various dissolutional patterns observed ultrastructurally in the mineral components of prisms and lamellae exposed in incomplete boreholes excavated by *Urosalpinx cinerea* in the valves of *Mytilus edulis*. The range of variation of solubility of different abiogenic mineral wafers, composed of elements commonly found as trace and minor elements in bivalve shell, in the secretion of normally functioning accessory boring organs, supports this hypothesis.

Snails left insoluble and moderately soluble minerals more frequently than soluble ones. This suggests that they are able to detect, probably by the odontophore, or possibly also with the heavily innervated accessory boring organ (Nylen, Provenza and Carriker, 1969; Carriker and Van Zandt, unpublished), the rate of excavation of the substratum. Thus, it appears that when, after a period of time, slight or no dissolution of the substratum occurs, a snail abandons it. The possibility also exists that some of the substrata could be chemically irritating or depressing, especially the relatively soluble sulfates.

Penetrability of mineral structures seems to bear no relation to hardness (Table I). Molluscan shell, for example, was excavated more deeply than strontianite, anhydrite, and witherite, all of which are softer than shell; and siderite, smithsonite, alunite, and fluorite, softer than shell, or at least about the same hardness as shell, were not penetrated at all.

The relative resistance of radular teeth to dissolution by the secretion of the accessory boring organ is not unexpected. After all, the radula is exposed to the secretion for relatively long periods of time during penetration. Furthermore, the success of boring gastropods over geologic time has unquestionably depended on the effectiveness of radular teeth in shell excavation. Although the radula functions in a secondary role in rasping and shaping the borehole (Carriker and Van Zandt, 1972b), the role is an obligatory one. Radular teeth are composed primarily of calcium, lesser amounts of strontium, and minor quantities of magnesium, boron, silicon, zinc, iron, and a series of trace elements (Carriker and Van Zandt, 1972a). Wafers of minerals containing iron, zinc, and silicon were unaffected by the secretion of the accessory boring organ. The possible insolublility of these minerals in radular teeth as well, and insolubility of the outer coating of the teeth, may contribute to the resistence of teeth to dissolution during the rasping process. An investigation such as carried out by Runham, Thornton, Shaw and Wayte (1969) on the mineralization and hardness of the radular teeth of the limpet could provide some very interesting information on this problem.

There is no association between degree of solubility of minerals in the accessory boring organ secretion and atomic number of the major cations in the minerals. Generally, with an increase in molecular weight of the major constituent, degree of dissolution decreased in the series shell, bone, teeth, strontianite, and witherite (anhydrite is a partial exception). However, molecular weights of several of the less soluble minerals (for example, magnesite, flourite, quartz) were considerably lower than those of the most soluble minerals.

The fact that beef bone was penetrated more rapidly than human tooth could have resulted from the presence of Haversian canals in the bone, allowing the secretion to penetrate more deeply than on a flat surface. The more rapid penetration of dentine than of enamel could also have been aided by tubules in the dentine (Fig. 7). The solubility product of bone and teeth is roughly comparable (Table I).

Differential dissolution of the core of enamel prisms by the secretion, leaving a substantial portion intact around the outside, suggests that the peripheral part of each prism is less soluble than the center. This is in contrast to dissolution of the shell of *Mytilus edulis,* in which the peripheral area of prisms precedes that of the axial component (Carriker, 1978). Examination of Figure 7 suggests, however, that, as in the case of dissolution of the prisms of *M. edulis,* the thin exterior organic envelope surrounding each enamel prism may also have been dissolved. The present study thus supports the initial results obtained by Carriker, *et al.* (1963), which suggested that excised accessory boring organs etch polished human enamel and dentine.

The capacity of *Urosalpinx cinerea* to penetrate a wider variety of minerals than we anticipated (Carriker and Smith, 1969), extends access to alternate potential foods in times of lack of the common diet, and enhances the probability of survival of the species. The ubiquity and success of the species undoubtedly reflects this capability.

This research was begun during our tenure in the Systematics-Ecology Program, Marine Biological Laboratory, and completed at the University of Delaware. It was supported in part by Public Health Service Research Grant DE-01870 from the National Institute of Dental Research, and in part by a grant from the University of Delaware Research Foundation. Thanks are expressed to Dr. Norman Starosta for providing the human teeth, Takako Nagasi for collaboration in the scanning electron microscopy, and Michael Castagna for generously collecting and shipping live snails from the eastern shore of Virginia, and Robert Biggs, Julius Gordon, John Milliman, Robert Palmer, and Robert Prezant for valuable suggestions on the inital draft of the manuscript. University of Delaware, College of Marine Studies Contribution No. 122.

SUMMARY

1. Results of an experimental study of the capacity of *Urosalpinx cinerea follyensis* Baker to penetrate 15 kinds of molluscan and abiogenic minerals are reported. Minerals, cut into small wafers, and radular teeth were exposed to penetration by normally boring snails in valve models. Depth of incomplete boreholes was measured with a compound microscope. Extent of dissolution was examined with a scanning electron microscope. All biogenically formed calcareous minerals, except radulae of *U. cinerea,* and some abiogenic minerals, were penetrated. Rate of penetration of wafers decreased in the following order: calcite

and aragonite (shell of *Crassostrea virginica, Spisula solidissima, Anomia simplex*), strontianite, bone and tooth hydroxyapatite, anhydrite, witherite, and magnesite. Abiogenic minerals siderite, smithsonite, alunite, fluorite, and quartz were not penetrated. Of the 15 tests performed on biogenically formed calcareous minerals, only two snails (or 13%) left the sample on the model before the experiment was terminated; of the 25 tests on abiogenically formed minerals, 20 snails (80%) crawled off the sample before the end of the tests. Experiments demonstrated that Ca, Sr, Ba, and Mg, as carbonates, and Ca both as the phosphate and sulfate, were attacked by the secretion. Fe and Zn, as the carbonates, Ca as the fluoride, Si as the oxide, and K and Al as the sulfate were unaffected.

2. Degree of penetration of beef bone was intermediate between that of shell and human teeth. Dentine was penetrated more deeply than enamel. The core of enamel prisms was dissolved more deeply than the outer region by the secretion. Radular teeth of *U. cinerea* were unaffected by the secretion during exposures ranging from 0.5 to 2.5 hr.

3. The capacity of *U. cinerea* to penetrate a wide range of types of minerals explains, in part, the large number of different species upon which it can prey.

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ORGANIZATION AND FUNCTION OF CTENOPHORE COLLOBLASTS: AN ULTRASTRUCTURAL STUDY

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The members of the phylum Ctenophora differ from those in the phylum Cnidaria in well defined characteristics, such as the occurrence of swimming comb rows, the presence of an ectomesoderm and the possession of special adesive cells termed colloblasts. These colloblasts are typically situated on the two tentacles of the Ctenophora, the only known exception being *Euchlora rubra,* in which the colloblasts are replaced by nematocysts (Tregouboff and Rose, 1957).

Early light microscope studies of colloblast structure (Komai, 1922, cited by Hyman, 1940; Weill, 1935a) were complemented by an electron microscope study performed by Hovasse and De Puytorac (1962). For these latter, the colloblast is a single epithelial cell, the apical pole of which (collosphere or colloblast head) is swollen by numerous eosinophilic granules. Each granule is at the end of a dense fibril which arises from a unique star-shaped structure named the "spheroidal body." This, situated in contact with the nucleus, was interpreted as a nuclear extrusion rather than as a centrosome, because the nuclear membrane was apparently absent at their point of contact. The nucleus of the colloblast stretches towards the basal pole of the cell and corresponds to the axial filament described with the light microscope. A second filament, interpreted as the "spiral filament" (helical thread) originates from the spheroidal body, reaches the cell surface, encircles the cell in two or three whorls and runs towards the fibrous axis of the tentacle. This helical thread was described as a non-fibrous tube, surrounded by the plasma membrane expanded into six to ten longitudinal anastomosing cristae (membrane folds). Some refractive vesicles ("brilliant granules") are also situated at the external surface of the colloblast head. A covering cell forms a cap above several colloblast heads and was interpreted as being responsible for transporting the colloblasts along the tentacle.

More recently, Bargmann, Jacob and Rast (1972) published a detailed ultrastructural study of the tentacle of a ctenophore. They observed the features of the colloblast cell described above, as well as new structures such as the bottle brush root ending of an anchoring apparatus and the existence of "procolloblasts" (precursor cells) arising from cells of the covering layer of the tentacle. A new interpretation of the origin of the outer refractive vesicles of the colloblast head was given; they could result from a very fast extrusion of the intracytoplasmic eosinophilic granules. However, their study also included some unusual observations, such as the absence of a nuclear membrane over great parts of the surface of the nucleus. In addition, the structure similar to the "spiral filament" of Hovasse and De Puytorac (1962) was considered to be a straight stalk. As the membrane folds surrounding this stalk had a slightly spiralized disposition, Bargmann *et al.* (1972) suggested it was these structures that had given rise to the erroneous description of a helical thread by previous authors.

The present study of four species of ctenophores was thus undertaken with a view to elucidating the structure of the colloblast in general and to resolve the above mentioned conflicting observations in particular. Also, new evidence is presented concerning the innervation of the colloblasts and the mechanisms of colloblast activation and adhesion.

MATERIALS AND METHODS

The study was performed on four species of the phylum Ctenophora (*Pleurobrachia rhodopis, Eucharis multicornis, Cestus veneris* and *Lampetia pancerina*) collected from the bay at Villefranche-sur-Mer (France).

For transmission electron microscopy (TEM), tentillae, tentacles and tentacle bases were fixed with 3% glutaraldehyde and 1% osmium tetroxide, in a 0.1 M sodium cacodylate buffer (pH 7.8). All media were adjusted to the osmolarity of sea water. After embedding in Epon, thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in Philips EM300 and Hitachi HU12A electron microscopes.

Specimens of *Pleurobrachia* were fed *Artemia* nauplius larvae (Crustacea, Branchiopoda) and tentacles and tentillae which had captured larvae were cut off, fixed and embedded as above.

Two preparations were used for scanning electron microscopy (SEM): first, isolated tentacles of *Pleurobrachia* and body fragments with their tentacular sheets, and secondly, tentacles of *Eucharis* which had "captured" glass coverslips. After double fixation, the specimens were critical-point dried using acetone-carbon dioxide, then sputter coated with gold and observed in a Cambridge S600 scanning electron microscope.

RESULTS

The tentaculate Ctenophora bear two very long contractile tentacles, situated on either side of the sagittal plane, which is determined by the pharynx of the animal. Many shorter and thinner contractile filaments occur along each tentacle and, in *Eucharis* and *Cestus,* around the lips. These constitute the tentillae (Fig. 1). Tentacles and tentillae have the same organization: an axis of muscle cells embedded in a dense fibrillar mesoglea (Franc, Franc and Garrone, 1976) within which lie the elements of the tentacular nerve, and a peripheral cortex consisting of epithelial cells, mucous cells, sensory cells and colloblasts. The colloblasts are more numerous on tentillae than on tentacles. The sensory cells each possess a non-motile cilium and sometimes also show sensory peg structures as described by Hernandez-Nicaise (1974b). They are grouped into clusters and are always associated with mucous cells. These sensory clusters constitute an extensive surveillance system over the surface of the widespread tentacular apparatus (Fig. 2).

Colloblast organization

Seen with SEM (Fig. 3) or TEM (Fig. 4), the mature colloblast is a single pear-shaped cell, anchored to the tentacular mesoglea by its slender basal end. The size of these cells varies with the species studied: the smallest are found in *Pleurobrachia* where they are 10 μ in length and a maximum of 4 μ wide, whereas in *Eucharis* the colloblast may reach a length of 25 μ and a width of 8 to 10 μ. However, the length also varies considerably depending on their degree of extension at fixation.

The colloblast always contains a nucleus which is elongated and whose form follows that of the pear-shaped cell (Fig. 4). It is surrounded by a typical nuclear membrane and contains evenly dispersed, thin, granular chromatin. No nucleolar structure was detected in the mature colloblast. Some mitochondria, occasional rough endoplasmic reticulum cisternae and few vesicles and microtubules are located in the cytoplasm.

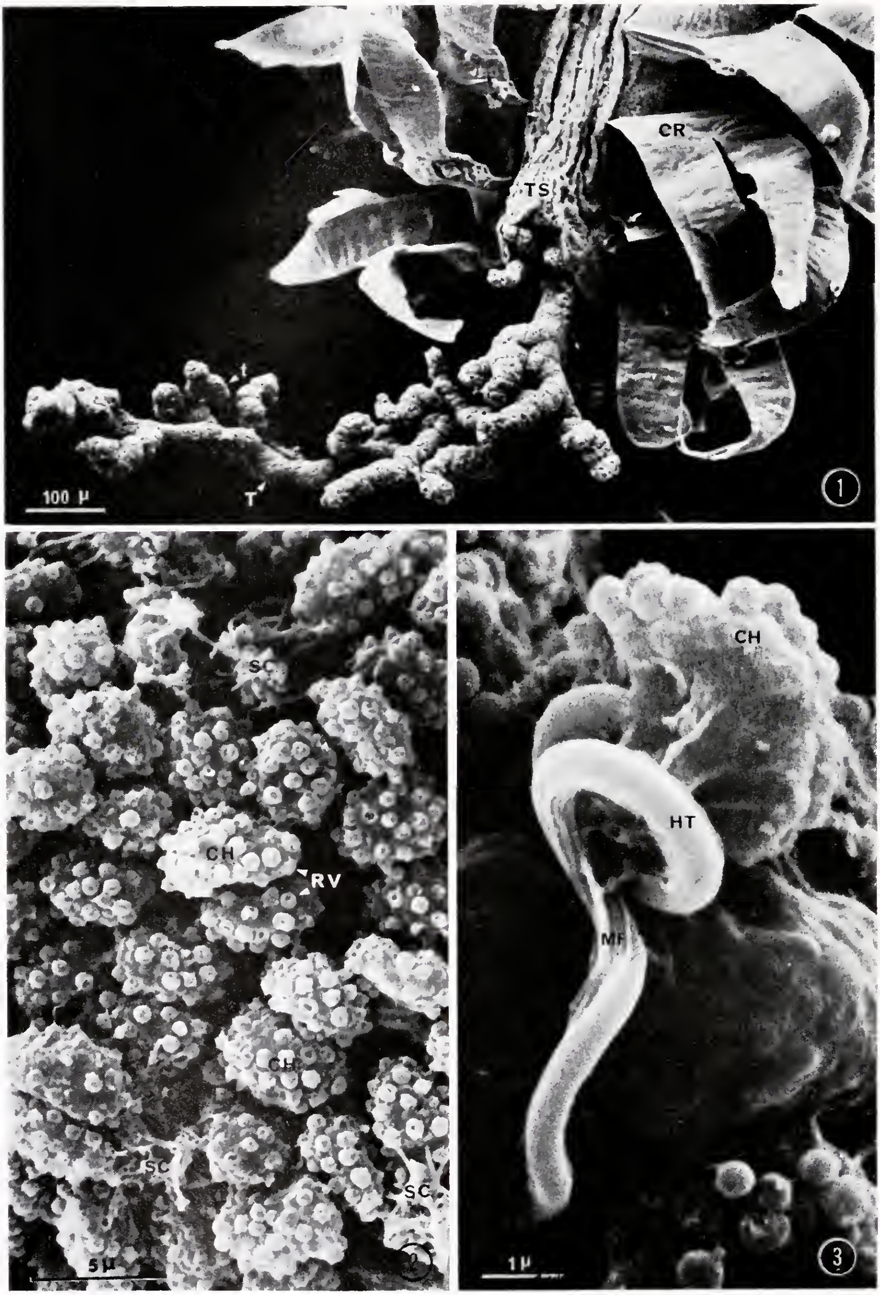
The expanded end of the colloblast contains numerous peripheral granules (Fig. 4). These correspond to the "eosinophilic granules" of light microscopy. They are membrane bound spherical vesicles about 0.8 μ in diameter, with finely granular contents and may show dense, secondary concentric or radial formations, in the mature state (Fig. 5). Above these granules, the colloblast plasma membrane bears a particularly well developed cell-coat with a hexagonal substructure (Fig. 6).

The colloblast head generally bears several indented vesicles (Figs. 2, 4, 5). Irregular in outline and containing variable amounts of electron dense material, these vesicles lie external to the colloblast membrane in the hollows between the eosinophilic granules. Neither connection of the collobast membranes with those of the external vesicles, nor evidence of extrusion of granules from the colloblast has been observed. By virtue of their position, these vesicles can be identified with the "refractive vesicles" of light microscopy.

Apart from the eosinophilic granules and the refractive vesicles, the principal feature of the colloblast cell is the presence of a helical thread. This intracellular structure extends from the base to the apex of the cell and is generally in the form of a right hand helix, but is occasionally left-handed. Although the helical thread is loosely coiled for the first two turns, it later tightens before heading towards the center of the colloblast head. The diameter (0.25–1.25 μ), length and number of turns of the helical thread vary with the origin of the colloblast. In *Lampetia* and in *Cestus* there are but one or two turns (Figs. 7, 8), whereas in *Eucharis* the thread is tightly coiled and there are six to seven turns of the helix (Fig. 9).

Where the helical thread follows the cell surface, it lifts the plasma membrane but remains connected to the cell body by a thin strip of cytoplasm (Fig. 10, arrows). Parallel to this strip, the membrane expands in five to eight folds which longitudinally follow the inner surface of the helical thread as far as its penetration into the colloblast head. Here, the folds of the membrane gradually diminish and then disappear. Along its invagination into the cell, the plasma membrane forms a double sheath around the end of the helical thread.

In section, the thread is heterogeneous. A cortex is well differentiated in *Pleurobrachia* and less easily seen in *Cestus* and *Eucharis*. The core can be


CR
TS
t
T
100 µ
1
SC
CH
RV
CH
SC
SC
5µ
2
CH
HT
MF
1µ
3

FIGURES 1–3. SEM of the tentacle of *Pleurobrachia rhodopis*.

FIGURE 1. View of a tentacle (T) with tentillae (t) emerging out of the tentacle sheath (TS). Comb rows (CR) are visible on either side of the sheath.

FIGURE 2. Surface view of a tentilla. The heads of the colloblasts (CH) bear outer

very electron dense (*Lampetia, Pleurobrachia*) or can present a lighter, finely granular structure (*Cestus, Eucharis, Pleurobrachia*). Within this core, six to nine peripheral simple longitudinal microtubules can be observed in the early stages of colloblast formation (Fig. 11).

The distal end of the helical thread terminates at the upper part of the nucleus and is differentiated into a structure commonly called the "spheroidal body". This consists of very dense material, in continuity with the cortex of the helical thread, and covering its distal end. This spheroidal body is set more or less in a hollow in the upper part of the nucleus which can even surround the distal end of the helical thread. However, the nucleus is invariably separated from the spheroidal body by a continuous nuclear membrane (Fig. 12). From the spheroidal body radiate fibrillar extensions, named radii, each of which terminates on one of the peripheral eosinophilic granule (Figs. 4, 7, 9, 12). At the point of contact, this radius is enlarged to produce a small attachment patch which bears a tuft of fine fibrils (Fig. 5). These are attached to a thickening on the outer surface of the granule membrane, on the inner side of which is a localized increase in density of the vesicle content. It is from here that the dense secondary formations in the mature eosinophilic granule arise.

At the opposite end of the helical thread is an electron dense, cone-shaped root structure. In *Lampetia,* it has a finely fibrillar organization, more or less packed in bundles with an axial periodicity of about 850 Å (Fig. 13). The surface of the root is bristled with numerous, thick filaments, giving it the appearance of a bottle brush (Figs. 13, 14). This root extends the colloblast plasma membrane into the intermuscular mesoglea of the tentilla. Here, the colloblast foot is enclosed in a thin jacket of extracellular material to which are attached massive fibers, specific to this mesoglea.

A synaptic junction always occurs in an expansion of the colloblast cell membrane, which is situated either at the basal region of the helical thread or just above the root structure (Fig. 14). The synaptic cleft is 100 to 125 Å wide. In the neurite ending, small electron-lucent vesicles 700 to 1000 Å in diameter line the presynaptic membrane. They are associated with a cisternum of the endoplasmic reticulum and a mitochondrion. However, often these latter two organelles are not visible; in which case, the synaptic junction is characterized only by the presence of the lining vesicles and the parallel array of the two plasma membranes. This synapse is always polarized in the same direction, towards the colloblast. Occasionally, the same neurite appeared to form synapses with several colloblasts or even with mucous cells and colloblasts.

Specialized epithelial cells constitute a continuous cover on the tentacle. Upon the tentilla, and possibly at the tentacular ends, these covering cells become irregular. They occupy the free spaces between the sensory elements, the mucous cells and the heads of mature colloblasts to which they are linked by a *zonula adherens.*

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refractive vesicles (RV), some of which are depressed. Three sensory complexes (SC) are seen breaking through the surface of the tentilla.

FIGURE 3. Higher magnification of a colloblast partially removed from the tentacle. The membrane folds (MF) running along the helical thread (HT) are visible due to partial disruption of the cell body. CH shows colloblast head.

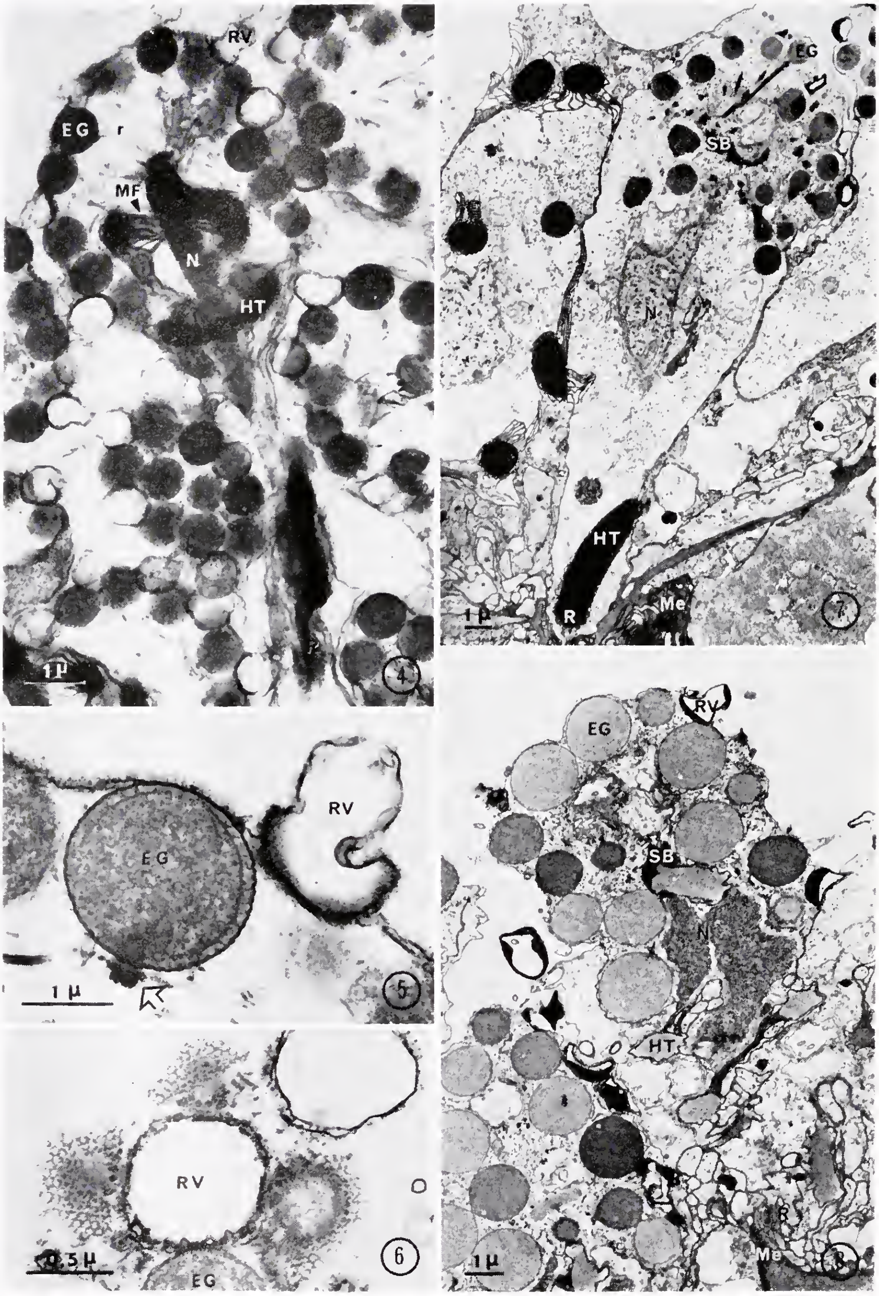
RV
EG
r
MF
N
HT
1 µ
4
RV
EG
1 µ
5
RV
EG
0.5 µ
6
EG
SB
N
HT
R
Me
1 µ
7
RV
EG
SB
N
HT
R
Me
1 µ
8

FIGURE 4. TEM of a thick section (0.5 μ) of a *Pleurobrachia* colloblast. EG shows inner eosinophilic granule; HT, helical thread; MF, membrane folds; N, nucleus; R, root; r, radius; RV, outer refractive vesicle.

Capture phenomenon

In their natural state, or removed from the animal, tentacles and tentillae consistently stick to a glass rod or an *Artemia* larva put in contact with them. This reaction will occur repeatedly with the same tentacle, but is evenutally abolished by too frequent touching with the glass rod and does not occur if the animal has its pharynx full of prey.

Comparing cross sections of tentillae originating from a resting *Pleurobrachia* and from a *Pleurobrachia* activiated by a captured *Artemia* larvae, two main differences appear. The first is the presence of a clear area between the musculo-mesogleal axis of the activated tentilla and its external sheet of colloblast heads. This area consists of the cytoplasmic extensions and loose helical threads of the colloblasts embedded in a finely granular mesoglea from which the massive fibers are completely absent. The second difference is the position of the sensory cells relative to the colloblast heads. In the resting tentillae, the colloblast heads are situated at the level of the sensory cells and, consequently, the sensory cilia protrude from the surface of the tentilla. In the activated tentillae, however, the colloblast heads extend beyond the sensory cells, which remain at their original level.

In SEM examination of tentacles attached to glass coverslips, the sticking phenomenon appears localized. At the contact points, the gluing substance covers the surface of the "prey" in those places where the colloblast heads lie. These may or may not be connected by threads to their tentilla. Often the helical thread is not visible and the wall of the foot seems uniform because of the presence of the covering cells. In other cases, the helical thread lies unfolded along the cell body, but complete extension of the helical thread is rare. Colloblasts not in contact with the "prey" appear unmodified.

In TEM examinations of tentillae which have captured an *Artemia* larva (Fig. 15), the crustacean cuticle bears a coarse, granular product in which are seen empty, membrane-bounded cavities with irregular outlines. Also present may be the remains of colloblast heads partly surrounded by their cystoplasmic membranes and containing some intact eosinophilic granules, as well as the anterior end of the helical thread and the more or less extended radii. The adhesive substance contains electron dense filaments, some of which converge onto the same point. Here, a structure similar to the small attachment patch of the eosinophilic granules is recognizable (Fig. 15, arrow).

Straightened helical threads are observed only on such preparations. The diameter and the inner structure of each thread remain identical to that of the

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FIGURE 5. Detail of an eosinophilic granule (EG) with its secondary dense formations and the attachment patch (arrow). RV shows refractive vesicle.

FIGURE 6. Tangential section of the cell-coat of the colloblast head. EG shows eosinophilic granule; RV, refractive vesicle.

FIGURE 7. Longitudinal section of a colloblast of a *Lampetia* tentacle. EG shows eosinophilic granule; HT, helical thread; Me, mesoglea; N, nucleus; R, root; SB, spheroidal body.

FIGURE 8. Colloblast from a buccal tentilla of *Cestus*. Legends as in Figure 7.

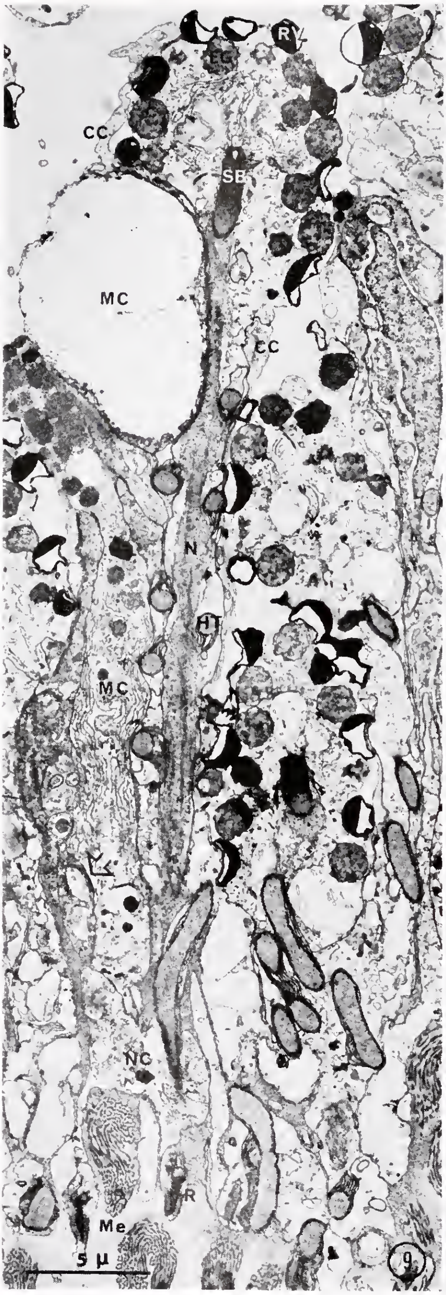
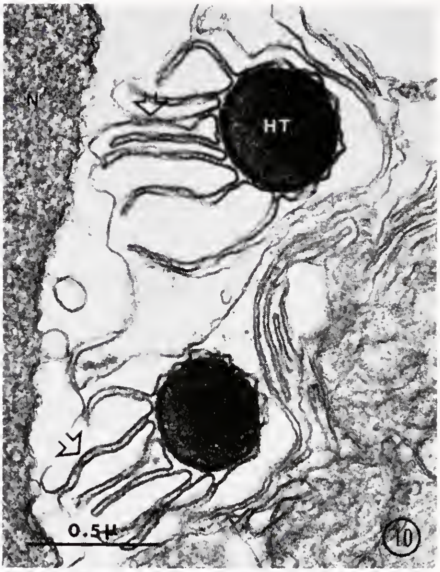
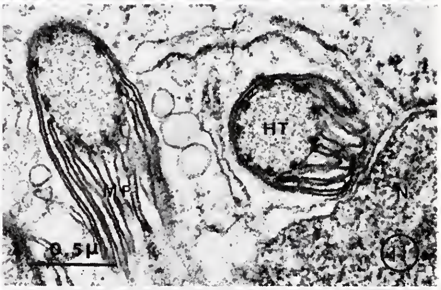
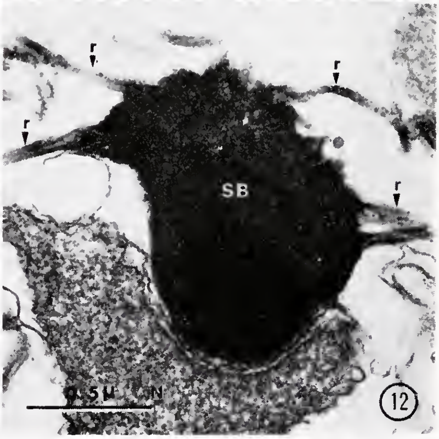

R
EG
CC
SB
MC
CC
N
HT
MC
NC
R
Me
5 μ
9

FIGURE 9. Longitudinal section of a colloblast of an *Eucharis* tentilla; note the nerve ending (arrow) connecting a mucous cell (MC). CC shows covering cell; EG, eosinophilic granule; HT, helical thread; Me, mesoglea; N, nucleus; NC, nervous cell; R, root; RV, refractive vesicle; SB, spheroidal body.




HT
Mf
N
0.5μ
11


r
r
r
r
SB
0.5μ
N
12

supposed resting colloblast, but cell shape and the arrangement of the membrane folds change. A marked reduction of the cell body diameter is seen corresponding to its increased length. In cross sections of the basal region of such a cell, the helical thread and sometimes the nucleus are visible, closely enveloped in the plasma membrane. The membrane folds are still present, but now are arranged in a loose helix around the straightened helical thread.

Discussion

The present study has included representatives of each order of the Tentaculata Ctenophora, except for the Platyctenea, which live mainly in warm waters (Hyman, 1940). Colloblasts from all the animals studied were found to be similar in structure, differing only in details such as their overall size or the length of the helical thread. This applies to both colloblasts borne on tentacular tentillae and those of buccal tentillae. The present observations confirm the unicellular nature of the colloblast throughout the ctenophores. Its generalized structure is given in Figure 16.

The scarcity of cytoplasmic organelles and the absence of a nucleolus indicate the low level of synthesis in the mature cell. However, no sign of degeneration was observed in either the resting or the active colloblast. The incomplete nature of the nuclear membrane described by Bargmann *et al.* (1972) may be a fixation artifact, as optimal fixation is often difficult in animals such as ctenophores which have a high water content.

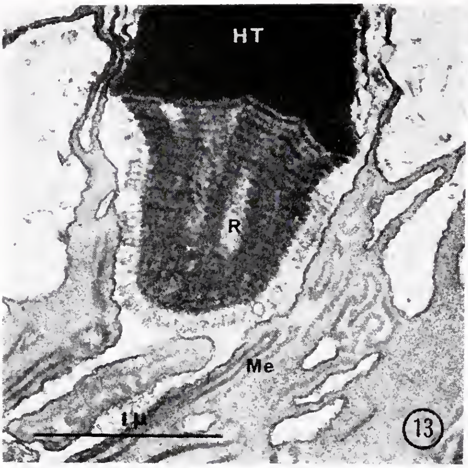
The ultrastructural examination of captured *Artemia* larva shows that the eosinophilic granules are responsible for the gluing action of the colloblast. The capture phenomenon involves the disruption of the colloblast. Many of the released eosinophilic granules burst, liberating their glue, which traps the prey and attaches it to the fibrous radii of the spheroidal body. Consequently, *the capture mechanism of the colloblast is self-destructive.* The outer refractive vesicles seem to play no part in the capture mechanism. This is in agreement with their weak adhesive properties (Weill, 1935b) and their irregular arrangement or absence from the surface of the colloblast head.

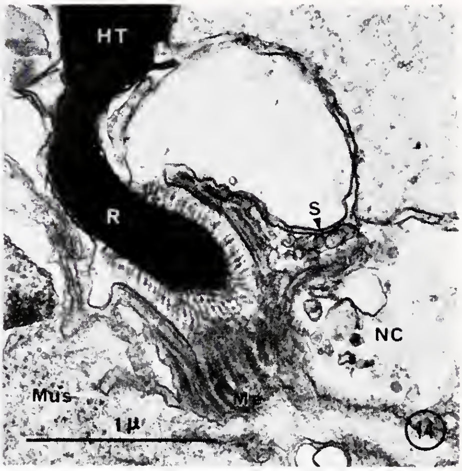
Bargmann *et al.* (1972) considered that the refractive vesicles are extruded eosinophilic granules whose contents have been rapidly transformed. Present observations do not support this interpretation. On the contrary, in an initial study on the histogenesis of the tentacular cortex (unpublished results), it was found that the refractive vesicles are the remains of an accessory cell which disappears during tissue differentiation.

FIGURE 10. Two transverse sections of the helical thread (HT) from a *resting* colloblast of *Pleurobrachia;* the cell membrane exhibits five to six folds as it wraps around the thread. Arrows show the thin strip of cytoplasm connecting this structure to the cell body; N, nucleus.

FIGURE 11. Two cross sections of a helical thread of an *Eucharis* immature colloblast. Note the nine peripheral microtubules. MF shows membrane folds.

FIGURE 12. Expanded distal end of the helical thread constituting the spheroidal body (SB) with fibrous radii (r) radiating from it; note the continuity of the membranes and space around the nucleus (N) and the close association between the spheroidal body and the outer nuclear envelope (*Pleurobrachia rhodopis*).





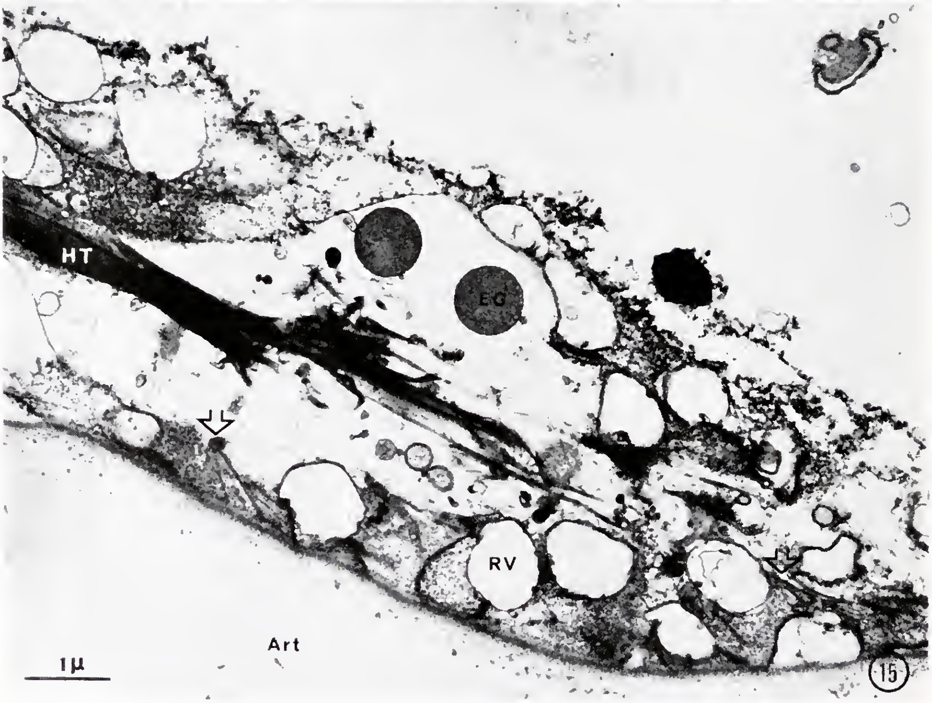


FIGURE 13. Longitudinal section of the colloblast root (R) in *Lampetia.* Note its periodic structure reminiscent of a ciliary rootlet. HT shows helical thread; Me, mesoglea.

FIGURE 14. Longitudinal section of the root (R) of a *Pleurobrachia* colloblast, embedded in the mesoglea (Me) between muscle cells (Mus). A polarized synaptic ending (S) makes contact with an expansion of the colloblast cell membrane. HT shows helical thread; NC, nervous cell.

FIGURE 15. Head of a *Pleurobrachia* colloblast adhering to the cuticle of an *Artemia* larva (Art). The burst eosinophilic granules have released a gluing substance in which attachment patches with the secondary denses formations (arrows) are still recognizable; some eosinophilic granules (EG) are still intact. HT shows helical thread; RV, refractive vesicle.

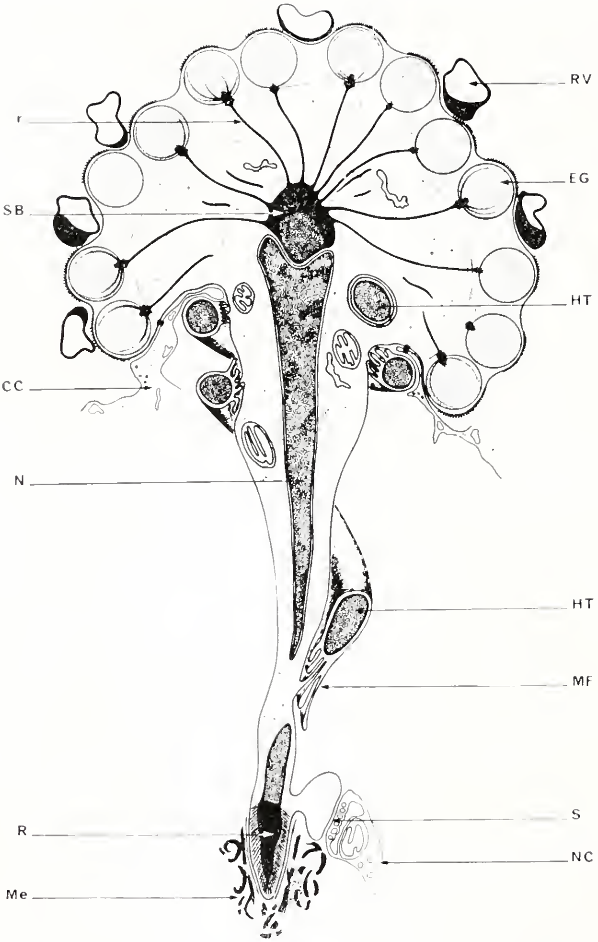


FIGURE 16. Schematic drawing of a resting colloblast as seen with the electron microscope; to emphasize its helical form, the thread is partly represented in shadowed perspective. CC represents covering cell; EG, eosinophilic granule; HT, helical thread; Me, mesoglea; MF, membrane folds; N, nucleus; NC, nervous cell; R, root; r, radius; RV, refractive vesicle; S, synapse; and SB, spheroidal body.

The helical form of the thread of the resting colloblast was demonstrated both by SEM and by TEM observations of thick sections of tentillae. This confirms the early light microscope studies of Komai (1922, cited by Hyman, 1940) and Weill (1935a), and the first electron microscopy description by Hovasse and De Puytorac (1962). However, Bargmann *et al.* (1972) considered that the "spiral filament" seen by light microscopy was in fact the helically arranged membrane folds surrounding a straight tube. This discrepancy can be explained as follows. In a *resting* colloblast, the membrane folds follow the helical thread along the generatrix line facing the longitudinal axis of the cell (Fig. 17a). But, in an *extended* colloblast, it is the membrane folds that form a loose helix around the straightened thread (Fig. 17b). This arrangement results

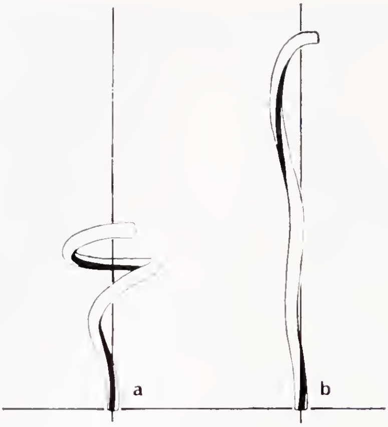


FIGURE 17. Relative positions of the membrane folds (black) on the thread (clear) in its helical (a) and extended (b) conditions.

from the relative movement between the membrane folds and the helical thread *without rotary motion of their ends* during extension of the cell. This is assured by the anchoring of the thread in the tentillar mesoglea and the *zonula adherens* junction binding the colloblast head to the surrounding covering epithelial cells. It is probably on colloblasts in this latter condition that Bargmann *et al.* (1972) based their interpretation.

No modification in diameter or content of the thread was observed between its resting or extended state. The various aspects of the helical thread matrix are observed on different tentillae of the same tentacle and may thus be related to the maturity of the cell, the clearer matrix corresponding to the most differentiated state.

The presence of microtubules at the periphery of the helical thread, the close association of its distal end with the nucleus and the ciliary root-like structure observed in *Lampetia* could suggest a ciliary origin for the helical thread. However there are significant differences between this thread and the well-known flagellar structures (Afzelius, 1969). For example, no basal body structure was ever seen in a mature colloblast, either at the root end or at the spheroidal body. The peripheral microtubules of the thread are not in a doublet or a triplet arrangement and no central microtubules are present. If not indicative of a ciliary origin, the peripheral microtubules may have a skeletal function (Pochon-Masson, 1967).

In spite of their close resemblance at the light microscope level, the helical thread differs from the protozoan peritrich ciliate stalk (Amos, 1972; Favard and Carasso, 1965; Huang and Pitelka, 1973) by the absence of microfilaments. The paucity of microtubules means that an analogy between the colloblast helical thread and the axostyle of certain protozoan flagellates (Grimstone and Cleveland, 1965) cannot be considered seriously. A similarity can only be established between the colloblast and prehensile tentacle of certain protozoan suctorians (Batisse, 1965, 1966; Rudzinska, 1965) on the basis of their function (prey capture) and mechanism (gluing).

It is evident that the helical form of the thread assures a resilient attachment of the prey to the tentacle. Curiously, this helical shape is found elsewhere only in the evaginated filaments of the cnidarian nematocysts, which also have a gluing function (Mariscal, 1974). It remains to be established *how* the colloblast contacts its prey. It could occur by contact of the prey with the colloblast or by projection of the colloblast head towards the prey. The differences observed between a resting tentilla and a stimulated one suggest that excitation of one (or a few) sensory cilia could cause the elevation of all the colloblasts borne on a tentilla, but not their destructive projection. From a functional point of view, the retracted position of colloblasts on a resting tentilla would expose the sensory cilia, whereas the elevated colloblasts would more effectively expose the potentially adhesive colloblast heads.

Although a more complete study is needed to answer this question, the discovery of colloblast innervation corroborates the hypothesis of an activation process. This synaptic junction is identical with those previously described in ctenophores (Hernandez-Nicaise, 1968, 1973). The presynaptic organelles which constitute the "presynaptic triad" were consistently found in the nervous compartment of the synapse and indicate that the colloblast acts as an effector. It must be stressed that each colloblast bears a polarized synapse, as do cnidarian nematocytes (Westfall, 1969, 1970). Hernandez-Nicaise (1974a) presented histological evidence that sensory cells are indeed neurons which can connect directly with effector cells, especially in the tentacular epithelium. However, these sensory cells themselves also receive neuro-sensory polarized synapses in which they are the post-synaptic elements (Hernandez-Nicaise, 1974b). Consequently, it is possible that a direct sensory-motor pathway occurs between a sensory cell and a colloblast. If this is so, then a functional homology between the cnidarian nematocyte and the sensory cell-collobast complex of ctenophores can be considered. To emphasize this, it is of interest to notice the close resemblance between the cnidocil-stereocilium apparatus of the nematocyte described by Westfall (1970) in a hydromedusan, and the cilium and peg differentiations of the ctenophore sensory cell (Hernandez-Nicaise, 1974a).

As the capture of prey causes the destruction of colloblasts, each can be used only once. Now colloblasts must therefore be continually formed. This occurs in the lateral thickenings of the tentacle base epidermis (Hyman, 1940). The colloblasts are well differentiated when they arrive on the tentacle. The only modifications found were maturation of the eosinophilic granules, clearing of the dense matrix of the helical thread and integration of the colloblast cell by the nervous system. In particular, the "procolloblasts" described by Bargmann *et al.* (1972) do not exist on the tentacle. On the other hand, the presence of the covering epithelial cells is confirmed. Initially having a protective function at the tentacular level, these cells later assure the continuity of the tentilla epithelium.

Experimentation was performed at the Station Zoologique of Villefranche-sur-Mer, Université Pierre et Marie Curie (Paris) and electron microscopy observations at the Centre de Microscopie Electronique Appliquée à la Biologie et à la Géologie, Université Claude Bernard (Villeurbanne).

The author expresses his gratitude to Ms. M. Nowak and Dr. Ph. W. Ledger for their help in the preparation of the English form of the manuscript, and thanks M. A. Bosch for his efficient photographic assistance. This work was supported by C.N.R.S. (L.A. no. 244).

SUMMARY

The fine structure of ctenophore colloblasts was examined using scanning and transmission electron microscopy.

The same structure was found consistently in the three largest orders of the main class of Ctenophora (Tentaculata).

The colloblast is a pear-shaped cell, firmly anchored in the tentacular mesoglea by its tapering base. This highly specialized cell is always nucleated and contains an intracytoplasmic helical thread which divides into numerous fibrous radii at its distal end. Each radius bears at its tip an eosinophilic granule containing a mucoid substance which, when released by contact with the prey, glues it to the tentacle. The outer refractive vesicles, which are interpreted here as the remains of another kind of cell, seem to play no part in the capture phenomenon.

The innervation of the colloblast by a chemical synapse is described and discussed with respect to functional mechanics and phylogeny.

The colloblast appears to be a disposable capture cell and a new scheme of its organization is proposed.

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FEEDING AND DIGESTION IN THREE ENTOSYMBIOTIC GRAFFILLID RHABDOCOELS FROM BIVALVE AND GASTROPOD MOLLUSCS

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The entosymbiotic rhabdocoels show considerable variation in the extent of their adaptations to life within other organisms and this is particularly evident in their nutritional physiology (Jennings, 1971; 1974a). In some species, exemplified in the family Umagillidae by *Syndesmis antillarum* and *S. franciscana* from the gut and perivisceral spaces of echinoids, the diet, gut and digestive physiology are vitually identical with those of free-living flatworms (Jennings and Mettrick, 1968; Mettrick and Jennings, 1969). The symbiotes feed on co-symbiotic protozoa which abound in their habitat; they do not abstract the host's food nor feed on its tissues to any appreciable degree and their presence has no recognizable adverse effects.

In other, more extreme, instances, exemplified in the Fecampiidae by *Kronborgia amphipodicola* from the haemocoel of amphipods, the entire gut is lost and the rhabdocoels apparently feed cestode-fashion by absorption through the body surface (Christensen and Kanneworff, 1964; 1965). The epidermal microvilli are longer and more numerous than in free-living flatworms (Bresciani and Køie, 1970), presumably as an adaptation to this mode of nutrition, and the rhabdocoels cause atrophy of the host's gonads and, eventually, its death.

Most other studies on entosymbiotic rhabdocoels have been morphological or taxonomic; these indicate, however, that while there are probably many intermediate conditions between the umagillid and fecampiid modes of nutrition, the bias lies toward the former because most known species have seemingly functional alimentary systems and a normal epidermis. The resemblance to the umagillid pattern, though, may well be only superficial. A decisive factor in umagillid nutrition is the occurrence of large numbers of co-symbiotic prey organisms; this is a characteristic feature of echinoderms (Hyman, 1955) but is not found to the same extent in molluscs, which are the next most favored hosts of entosymbiotic rhabdocoels. Mollusc-rhabdocoel symbioses are of particular interest, since the monogenetic and digenetic trematodes are believed to have evolved from rhabdocoel-like ancestors (Hyman, 1951; Llewellyn, 1965; Baer, 1971) and the Digenea retain initimate associations with molluscs during their larval stages. Further, both Monogenea and Digenea possess functional alimentary systems as adults; they utilize their hosts' ingesta or tissues as food and show concomitant modifications of digestive physiology (Jennings, 1968). Nothing is known, though, of the nutrition of those rhabdocoels entosymbiotic with molluscs, or of how it compares with that of other rhabdocoels and of larval and adult trematodes. To remedy this, three closely related species from the family Graffillidae, two entosymbiotic in bivalves and one in gastropods, have been examined with particular reference to their diet, feeding behavior, gut structure and digestive processes. These features were found to be considerably influenced by the same aspects of the hosts' nutritional physiology and consequently these, too, have been studied and are reported on where relevant.

MATERIALS AND METHODS

The graffillid species studied were *Paravortex scrobiculariae* (Graff), *P. cardii* Hallez and *Graffilla buccinicola* Jameson. *P. scrobiculariae* was obtained from the intestine and digestive gland of the bivalve mollusc *Scrobicularia plana* (da Costa), collected bimonthly during 1976 and 1977 from mud banks near high water mark in the estuary of the River Esk, Whitby, Yorkshire; *P. cardii* was taken from the digestive gland of the bivalve *Cerastoderma edule* (L.), collected in October and November 1975 from around low water mark on sandy shores at Spurn Head, Yorkshire and at irregular intervals during 1975–77 from similar habitats at Plymouth, Devon; and *G. buccinicola* was obtained from the digestive glands of the gastropods *Buccinum undatum* L. and *Neptunea decemcostata* (Say). *B. undatum* was collected during December 1977 and January 1978 from 20 to 30 m of water off the Isle of Cumbrae, Scotland and *N. decemcostata* in July 1977 from 20 m in Cobscook Bay, Eastport, Maine.

Specimens of *S. plana* and *C. edule* were fixed in marine Bouin's fluid or 10% neutral formalin as they were collected during the intertidal period, for subsequent histological examination of the rhabdocoels *in situ* within their hosts. Section of the adductor muscles facilitated penetration of the fixatives. Others were dissected in sea water and the rhabdocoels fixed separately in marine Bouin's, 10% neutral formalin, Susa, 90% ethanol or Flemming's fixative. Living bivalves were maintained in circulating sea water in Leeds for periods of up to four months and used in feeding experiments in which suspensions of rice starch grains, yeast cells stained with Congo red, or bacteria isolated from sea water and cultured on seawater-nutrient agar were introduced into their inhalent currents. Such fed bivalves were then either fixed in marine Bouin's or 10% neutral formalin, or dissected and the rhabdocoels fixed, at progressive intervals up to 24 hr after feeding. Parallel observations were made on living rhabdocoels from fed hosts, examined by bright field, dark ground and polarized light illumination systems or after intravital staining with 0.05% seawater solutions of Neutral red or Janus green.

The gastropod *B. undatum* was maintained in circulating sea water until required; individuals were then dissected and either the entire digestive gland or rhabdocoels removed from this fixed, as above. Only preserved material from *N. decemcostata* was available; this was an intact digestive gland fixed in marine Bouin's six hr after collection of the gastropod, and was kindly supplied by Mr. Carter Newell of the Cobscook Bay Marine Laboratory.

Histological and histochemical observations on the gut contents, gut structure, food reserves, sequence of digestion of the natural and experimental foods, and the identity, origin and sites of action of the digestive enzymes were made on 4 or 8 μm serial sections of appropriately fixed materials and on whole mounts of individual flatworms. Stains and techniques used included: Ehrlich's haematoxylin and eosin; Heidenhain's haematoxylin and metanil yellow; Mallory's triple stain; Nuclear Fast red; Feulgen's reaction for DNA; Steedman's Alcian blue method for acid mucopolysaccharides; the periodic acid-Schiff (PAS) reaction for carbohydrates and acid mucopolysaccharides; Best's stain for glycogen; the Sudan IV and Oil red O methods for lipids; Twort's modified Gram stain; the Cyanol blue method and a modified diaminobenzidine technique (Phillips, 1978) for haemoglobin; Perl's method for inorganic iron and the alizarin-ammonium hydroxide method for inorganic iron and calcium.

For histochemical studies of digestive enzymes, fixation was at 1° C in 10% formalin buffered with phosphate to pH 7.0. Whole mounts, serial sections prepared after dehydration in graded acetones and impregnation in paraffin wax (melting point, 39° or 45° C) and frozen sections were treated by the indoxyl acetate method for non-specific esterases (Holt, 1958), the L-leucyl β-naphthylamide hydrochloride method for arylamidases (Burstone and Folk, 1965), the naphthyl AS-BI phosphate methods for acid and alkaline phosphatases (Burstone, 1958), the post-coupling 6 bromo-2-naphthyl-β-D-glucopyruronoside (glucuronide) method for β-glucuronidase (Pearse, 1972), the 6 bromo-2-naphthyl-β-D-galactopyranoside method for β-galactosidase (Pearse, 1972) and the naphthol AS-nonanoate method for lipase (Abe, Kramer and Seligman, 1964). Esterases demonstrated by Holt's method were characterized further, using specific inhibitors and activators and following procedures and interpretations given by Pearse (1972) and Hassall and Jennings (1975).

Positive controls for the enzyme studies consisted of simultaneous processing of appropriate mammalian and molluscan tissues; negative controls consisted of heat inactivated sections (held at 90° C for 2 min before processing) and the omission of specific substrates from incubation media.

Observations and Results

Paravortex scrobiculariae

70.4% of the *Scrobicularia plana* examined contained *Paravortex scrobiculariae;* the overall mean infection rate was 6.1 rhabdocoels per host with a range of 0 to 77 and a variance of 0.027. No seasonal fluctuations in the rhabdocoel population could be discerned and adults with embryos in all stages of development were found throughout the year. The species is hermaphrodite and viviparous; thin-walled capsules usually containing two developing embryos and a mass of yolk cells are given off from the female atrium into the mesenchyme. Mature individuals contain up to 40 capsules with embryos in different stages of development (Fig. 1). Fully developed embryos, resembling tiny immature adults, were seen to leave their capsules and move freely about the parental mesenchyme before passing through the gut wall into the intestine (Fig. 3). Birth is presumably via the mouth, in the primitive turbellarian fashion, but this was not observed.

Structure of the gut. The gut in *P. scrobiculariae* has the morphology typical of dalyellioid rhabdocoels (Fig. 2). The anterior subterminal mouth opens ventrally through a small ciliated buccal cavity into a doliiform muscular pharynx which lacks both intrinsic and extrinsic gland cells. A short (50–60 μm) ciliated oesophagus, composed of a single layer of cuboidal cells, links the pharynx with the simple saccate intestine. This occupies about two-thirds of the body length and is median in immature individuals, but in older ones is compressed and displaced laterally or dorsally by the vitellaria and developing embryos. The intestinal wall, or gastrodermis, (Figs. 2, 3, 7) is a single layer of monotypic broadly columnar cells, 75 to 100 μm tall and 30 to 50 μm wide, with basal nuclei and cytoplasm usually containing up to four large vacuoles. Gland cells of the type common in other Turbellaria are absent.

The gastrodermal vacuoles contain a clear proteinaceous fluid in which there is usually suspended a variety of spherical or granular inclusions. Most of these resemble in appearance and histological and histochemical reactions inclusions in the digestive cells of the bivalve host. In particular, many show the intense positive reactions for inorganic iron and calcium which are a characteristic feature of host digestive cell inclusions at certain stages of the host digestive cycle (Figs. 4, 5). Other, smaller inclusions very near the limit of resolution of the optical microscope show birefringence when viewed by polarized light.

The vacuoles may so distend the gastrodermis that opposing cells come together and occlude the lumen at irregular intervals, resulting in an intestinal structure dominated by large intra- and intercellular spaces. The latter, and the entire lumen when present as an uninterrupted entity, often contain the same materials as the intracellular vacuoles. This is particularly common in rhabdocoels from hosts fixed at low water.

The early embryonic gut consists of a pharynx and an undifferentiated mass of endoderm and yolk. By the time embryos are ready to leave their capsules and invade the parental mesenchyme (indicated by their continual rotation within the capsule by the beating of the epidermal cilia) the gut is organized into the adult form. The intestinal cells are vacuolated; the vacuoles have inclusions, but these are restricted usually to minute birefringent granules of the type seen in adult gut cells.

The food and feeding behavior. The diet, feeding behavior and digestive physiology of *P. scrobiculariae* are all greatly influenced by the feeding and digestive cycles of the host bivalve, especially the regular cycle of growth, food uptake, intracellular digestion, and disintegration undergone by the cells lining the distal tubules of its digestive gland. These cycles, which in turn are related to tidal rhythms and the duration of submergence at high water, must therefore be described. Only such detail as is necessary for understanding of the nutritional physiology of *P. scrobiculariae* will be given, however, as the cycles conform to the general pattern characteristic of intertidal bivalves as interpreted by Owen (1966; 1972; 1974) and reviewed by Purchon (1977). In particular, the digestive cycle closely resembles that described in *Lasaea rubra* by McQuiston (1969), who extended and reinterpreted the original observations on this species made by Morton (1956) and Morton, Boney, and Corner (1957).

Feeding and digestion in the host Scrobicularia plana. S. plana lives 15 to 20 cm deep in mud near high water mark and is submerged for 2 to 3 hr in each 12 hr tidal cycle. Specimens were occasionally found near mid-tide level or lower; these, obviously, were submerged for longer periods per cycle and did not show as clearly the cyclical changes in the digestive gland observed in those from the upper shore.

The bivalve is primarily a deposit feeder (Hughes, 1969) but is also capable of sustained suspension feeding. In the laboratory this facilitated the introduction of test foods into the inhalent current. The organic deposits forming the natural food of the population sampled were rich in bacteria, diatoms, protozoa, unicellular algae, and organic detritus; virtually all of these items contained much loosely bound iron and calcium which were easily demonstrated by the Perl's and alizarin techniques and formed, therefore, convenient labels of ingested natural foods throughout the digestive cycle.

The bivalve feeds while submerged at high water. Specimens collected as the tide receded, or from appropriate laboratory simulations using test foods, had well developed crystalline styles and full stomachs whose organic contents showed extensive extracellular digestion. The laboratory-fed specimens showed that this is effected by non-specific esterases, A- and C-esterases, β-glucuronidase, and arylamidases. Silt particles, and yeast cells or starch grains taken in excess by laboratory-fed specimens, were being passed into the intestine, which was filled with such rejected materials being transported by ciliary action to the anus. Semi-digested soluble and finely particulate organic materials were passing from the stomach through the main and secondary ducts of the digestive gland into the blind-ended distal tubules; the amount passing increased until around the time of low water, when the stomach was usually empty. The style in such specimens varied considerably, being absent from some and moderately to well-developed in others.

Shore-fixed specimens showed that there is some absorption by the unciliated epithelium of the secondary ducts during the passage of semidigested materials into the tubules, the epithelial cells developing transient reactions for iron and calcium.

The cycle of growth, intracellular digestion and disintegration undergone by the digestive cells of the tubules was found to be diphasic; all cells within a given tubule are in synchrony, but at any one time approximately half the tubules are in one stage of the cycle, while the other half are in the opposing stage. A complete cycle from juvenile stage to total disintegration occupies 24 hr, the stages are correlated with tidal (and hence feeding) cues and the disintegration phase coincides, roughly, with low water.

The mature digestive cells lining the fully developed tubules at high water contain many spherical vesicles, 2 to 10 μm in diameter, which show strong positive reactions for acid phosphatase, β-glucuronidase, β-galactosidase, non-specific esterases, A- and C-esterases, lipase and arylamidases. These are presumably primary lysosomes. Materials entering the digestive tubules from the stomach are rapidly taken up by these cells; occasionally fragments of algal cells, starch grains or intact bacteria could be recognized in the cells, but usually the engulfed materials were homogeneous, indicating extensive extracellular digestion in the stomach and tubule lumen. Vesicles 0.2 to 10 μm in diameter and showing in naturally-fed specimens strong reactions for iron and calcium appear in the digestive cells in increasing numbers. These are obviously newly formed food vacuoles or phagosomes; they show at first only moderate enzymic activity equivalent to that shown by the food as it enters the digestive gland from the stomach but quickly develop the intense reactions seen in the primary lysosomes. These include reactions for lipase and β-galactosidase, enzymes not demonstrated in the stomach. Eventually all vesicles in mature digestive cells show strong enzymic reactions, and the majority also show reactions for iron and calcium, indicating fusion of the primary lysosomes and phagosomes to form heterolysosomes within which intracellular digestion proceeds. Immature cells in other tubules show all types of vesicles, usually in smaller quantities and with less intense reactions for iron and calcium.

Intracellular digestion in the mature cells is completed around the time of low water; the heterolysosomes have shrunk as digested products are absorbed from them, but they contain one or more residual bodies staining intensely for iron and calcium and still showing much enzymic activity. Such cells disintegrate and the recognizable fragments, dominated by the residual bodies, pass down the tubules, through the secondary and main ducts (Fig. 5) and enter the stomach. A minority may pass directly into the intestine. Developing cells lining tubules in the opposing phase often still show intracellular digestive activity; these cells, of course, rapidly become the mature cells which will undertake the bulk of the intracellular digestion of the food to be taken during the next highwater feeding stage.

Tubules which have lost their lining of digestive cells are at this stage balloon-like structures with membranous walls. The source of the replacement epithelium was not seen; the junctions of the tubules and secondary ducts are lined by small cuboidal basophilic cells and these might give rise to the next generation of digestive cells.

Diet and feeding behavior in P. scrobiculariae. P. scrobiculariae feeds partly on semi-digested components of its host's food, which it abstracts while this is passing from the stomach into the digestive gland, and partly on the residual bodies and cellular debris released by the disintegration of mature host digestive cells at the end of their digestive cycle.

At high water the rhabdocoels are to be found almost exclusively in the anterior portion of the host's intestine, where they occur either singly or in groups of up to 10 or more (Fig. 6). The intestine is usually filled with silt particles, sand grains and other particulate materials, bound into a column with mucus, which have been rejected by the stomach's sorting mechanism and are being moved by the cilia of the intestinal epithelium towards the anus for defaecation. The rhabdocoels lie between the column and the intestinal epithelium and are always orientated with their ventral surfaces applied to the latter.

Previous accounts have described *P. scrobiculariae* only from the intestine (Villot, 1879; Wahl, 1906). Examination of hosts collected on the ebb tide, however, revealed that many of the rhabdocoels migrate anteriorly at this time from the intestine and pass through the main ducts of the digestive gland into the secondary ducts (Figs. 4, 5). A few penetrate almost into the digestive tubules proper but none were ever seen actually within the expanded distal portion of the tubules. Similarly, they were only rarely seen in the stomach, except around the intestinal groove and the bases of the main ducts of the digestive gland; such individuals were presumably in transit to or from the gland.

The rhabdocoels remain in the secondary ducts over low water but migrate

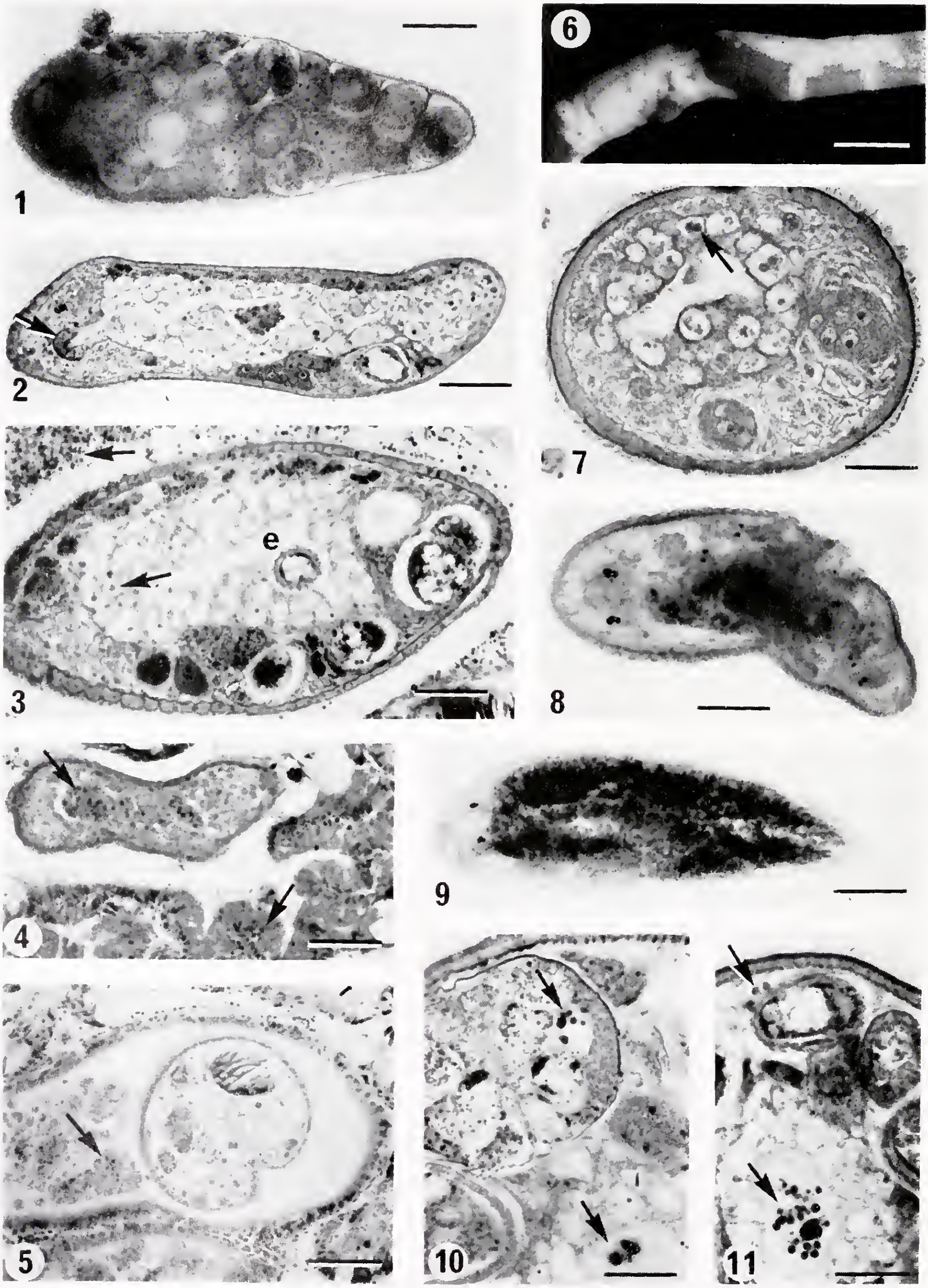

FIGURE 1. *Paravortex scrobiculariae.* A whole mount preparation treated by the cyanol blue method and showing a strong reaction (black) for haemoglobin around the brain and pharynx at the broad anterior end of the animal. The specimen contains many pairs of

back to the anterior intestine during the flood tide. While in the digestive gland they ingest materials present in the ducts; in the earlier part of their stay this consists mainly of the host's own semi-digested food passing up the ducts to the digestive tubules. This was proved by the demonstration of intact and partially digested yeast cells, starch grains and bacteria in the gut cells of *P. scrobiculariae* from the ducts of *S. plana* fed on these materials during a simulated highwater submergence (Fig. 7). In the later part of their stay, however, the ducts contain

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embryos within capsules in the mesenchyme; some, with prominent eyes, are at an advanced stage of development. Rupture of the specimen during preparation allowed partial extrusion of an early embryo (top left) through the body wall. The scale bar represents 0.25 mm.

FIGURE 2. *P. scrobiculariae.* A near sagittal section through a mature individual showing the pharynx (arrow), oesophagus, intestine and the intestinal wall or gastrodermis. The section is stained with Heidenhain's haematoxylin and metanil yellow; the scale is 0.2 mm.

FIGURE 3. *P. scrobiculariae.* A transverse section through the middle of the body showing an embryo (e) lying within the parent's intestine and others still in their capsules in the mesenchyme. The specimen was fixed *in situ* in the intestine of a *Scrobicularia plana* fed in the laboratory on a dense suspension of yeast cells; these (arrows) can be seen in the lumen of the host's intestine and within the intracellular vacuoles of the rhabdocoel's gastrodermis. The section is stained with Heidenhain's haematoxylin and metanil yellow; the scale is 0.1 mm.

FIGURE 4. *P. scrobiculariae.* An oblique longitudinal section through an immature specimen lying at the junction of a secondary duct and a digestive tubule, within the digestive gland of a *Scrobicularia plana* fixed at low water. The digestive epithelium of the tubule consists of mature cells (lower right) which contain iron-rich vesicles (arrow); similiar vesicles can be seen in the rhabdocoel's intestine (arrow). The section was treated by Perl's method for inorganic iron and is counterstained with Nuclear Fast red. The scale is 0.5 mm.

FIGURE 5. *P. scrobiculariae.* A transverse section through an individual lying in the lower part of a secondary duct of the digestive gland of a *Scrobicularia plana* fixed at low water. The duct contains, in addition to the rhabdocoel, cellular debris and iron-rich vesicles (arrow) from disintegrating digestive cells. Preparation and staining of the section were the same as for Figure 4; the scale is 0.5 mm.

FIGURE 6. *Scrobicularia plana.* The anterior portion of the intestine of an individual fixed at simulated high water and containing many *P. scrobiculariae* which are lying between the central core of silt particles and the intestinal wall. The rhabdocoels are all oriented with their ventral surfaces to the wall and hence their eyes, which lie dorsally just below the body wall, are not visible. The scale is 2.0 mm.

FIGURE 7. *P. scrobiculariae.* A transverse section through a mature individual, with well developed ovaries, fixed *in situ* within a secondary duct of a *Scrobicularia plana* fed on yeast cells during a simulated high water period. The duct (not shown) contained many intact and partially digested yeast cells; some of these had been ingested by the rhabdocoel and can be seen in intracellular vacuoles (arrow) within the gastrodermis. The section is stained by Twort's modified Gram stain; the scale is 0.1 mm.

FIGURE 8. *P. scrobiculariae.* A whole mount preparation of an individual dissected from its host and fixed after being kept for four days without food in filtered seawater. The intestine shows relatively slight reactions for A- and C-esterases, as compared with the specimen shown in Figure 9, and no reactions are visible in the intestines of the embryos. The preparation was treated by Holt's (1958) method for non-specific esterases, modified for the demonstration of A- and C-esterases as described by Pearse (1972) and Hassall and Jennings (1975). The scale is 0.25 mm.

FIGURE 9. *P. scrobiculariae.* A whole mount preparation of an individual fixed immediately after removal from a secondary duct of the digestive gland of a naturally-fed *Scrobicularia plana.* The intestine shows a high degree of reactivity to the technique for A- and C-esterases. Treatment of the preparation was the same as for that shown in Figure 8; the scale is 0.25 mm.

only the residual bodies and cellular debris from the disintegrating host digestive cells; these are passing down the ducts to the stomach and the rhabdocoels ingest quantities of these materials before returning to the intestine. This was apparent from the appearance in the rhabdocoels' intestine and gastrodermal cells of residual bodies staining heavily for iron and calcium (Fig. 4).

Ingestion is effected by a combination of ciliary and muscular activity. Cilia fringing the mouth and lining the buccal cavity sweep small particles and fluid into the mouth; contractions and expansions of the doliiform pharynx supplement the ciliary action and also draw in larger particles such as digestive cell nuclei and residual bodies. Rhabdocoels, isolated from the host and starved for up to 4 days in filtered sea water, fed in this way on yeast and bacterial suspensions but only small amounts were ingested and it was impossible to maintain the animals *in vitro* for any longer period.

The rhabdocoels are very rarely found in any part of the digestive gland during the period of high water and active feeding by the host. Conversely, though, some can always be found in the intestine of an infected host over the mid-ebb to mid-flow tidal period when others are actively feeding in the digestive gland. Such individuals contain little that is recognizable in their intestines apart from a few iron- and calcium-rich residual bodies. It is concluded, therefore, that *P. scrobiculariae* does not necessarily migrate to the digestive gland, and feed, during each tidal cycle but may remain in the intestine over one or more cycles to digest a meal taken earlier.

The host's intestinal contents occasionally include residual bodies from the digestive gland and it is conceivable that *P. scrobiculariae* may ingest these during its stay in the intestine. It is certain that feeding can occur in the intestine under laboratory conditions; when yeast cells, bacteria and starch grains were fed in excess to the host, many passed directly to the intestine and within a few minutes some had been taken up by rhabdocoels lying in the intestinal lumen (Fig. 3). Under natural conditions, though, the host's intestine usually contains very little potential food and it seems likely that feeding normally occurs predominantly, if not entirely, in the ducts of the digestive gland, following migration from the intestine during the period between successive high tides.

Digestion and food reserves. Serial sections and whole mounts of *P. scrobiculariae* fixed after being kept in filtered sea water for 4 days subsequent to their removal from the host showed that the gastrodermal cells contain only small amounts of endogenous enzymes. These are non-specific esterases, A- and C-esterases, acid and alkaline phosphatases, lipase and traces of β-glucuronidase

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FIGURE 10. *Paravortex cardii.* Part of a transverse section of an individual removed from a main duct of the digestive gland of a *Cerastoderma edule* fed intermittently on yeast cells over a period of three hours' total submergence. Yeast cells (arrows) can be seen in the intestinal lumen of the adult rhabdocoel and also in the intestine of an encapsulated advanced embryo. The section is stained with Heidenhain's haematoxylin and metanil yellow; the scale is 0.05 mm.

FIGURE 11. *P. cardii.* Part of another transverse section of the same individual used for the preparation seen in Figure 10. Yeast cells (arrows) can be seen in the intestinal lumen of the adult, as before, and also within an embryo's capsule, lying in the space between the capsule's wall and the embryo. The section is stained as for Figure 10; the scale is 0.1 mm.

(Fig. 8). No reactions could be obtained for β-galactosidase or arylamidases. The reactions for the other enzymes named were diffused throughout the cytoplasm and, so far as could be discerned with the light microscope, were not localized within lysosomes of the size and type seen in *Scrobicularia*. The pharynx and oesophagus are devoid of demonstrable enzymic activity, other than acid phosphatase in the intrinsic and extrinsic muscles of the pharynx.

No significant quantitative changes in enzymic activity could be demonstrated in *P. scrobiculariae* fed *in vitro* on starch grains or boiled yeast and bacterial suspensions, irrespective of the time elapsing between feeding and fixation. Increased non-specific esterase activity was found after feeding living bacteria but such activity clearly resided in the ingested food.

In marked contrast, rhabdocoels fixed during their stay in the ducts of the host digestive gland showed greatly enhanced enzymic activities (Fig. 9). These were localized in the inter- and intracellular vacuoles of the gastrodermis and their development coincided with the appearance at the same sites of vesicles and particles recognizable histologically and histochemically as originating either in the host's food or its digestive cells. This indication that the bulk of the enzymes effecting digestion of the food in *P. scrobiculariae* are, like the food itself, derived from the host, was confirmed by the occasional detection of β-galactosidase in the vacuoles. This enzyme was never detected in unfed rhabdocoels.

Enzymic activity continues at this elevated level for as long as visibly recognizable food remains in the gut vacuoles. It then fades to the level seen in unfed specimens and this diminution of activity coincides with the disappearance of reactions for iron and calcium. This latter disappearance implies that the rhabdocoel is capable of breaking down the residual bodies of the spent heterolysosomes of the host; those entering the host's stomach, in contrast, appeared to resist breakdown, but this point could not be confirmed.

Small quantities of lipid occur in the gastrodermis as droplets 0.3 to 1.0 μm in diameter. Larger amounts occur in the vitellaria, but these are obviously destined for inclusion with the fertilized eggs as energy stores for the developing embryos and do not constitute a normal long-term reserve for the adult.

The gastrodermal cells mesenchyme and vitellaria contain significant amounts of glycogen; no quantitative data were obtained but the strong reactions at these sites with the Best's carmine and PAS techniques indicated formation and storage of relatively large amounts.

Nutrition of the embryo. The developing embryo utilizes the lipid reserves of the yolk globules and these are exhausted by the time that the pair of embryos within each capsule are fully developed with eyes, nervous system and an organized gut. As the lipid reserves disappear, tiny birefringent granules accumulate in the vacuoles of the gut cells; these appear to be endproducts of lipid metabolism and are seen in adult gut cells also, but could not be identified. There was some evidence that once the yolk reserves are exhausted, and before the embryos leave their capsule, nutrients are passed from the parental gut through the capsule to the embryos. In a number of instances, inorganic iron was demonstrated in the gut cells of well developed embryos within adults from *Scrobicularia* fixed on the shore. The reaction was diffuse, resembling that seen in the epithelium of the secondary ducts of the host's digestive gland, and could have resulted from materials ingested by the rhabdocoel being passed directly to the embryos. Similar diffuse reactions for iron were occasionally seen in the gut cells of adults containing such embryos.

Support for this idea of supplementary nourishment of the embryos prior to their leaving the capsule came from the observation of yeast cells and fragments of starch grains, identified by their staining reactions with Twort's modified Gram stain, PAS and iodine, within the capsules and on occasion within the embryonic gut. An identical situation was observed in *Paravortex cardii* (Figs. 10, 11). The capsule wall appears to remain intact in such instances and the mechanism of transfer of soluble and particulate nutriments from the parental gut remains unknown.

Embryos which had reached the parental gut were sometimes seen to have recognizable materials such as residual bodies of host origin and yeast cells in their gut vacuoles, but these could well have been ingested after the embryo had invaded the adult gut. Late embryos released from their capsules in squash preparations swam freely and showed the pharyngeal contractions seen in feeding adults.

Haemoglobin. P. scrobiculariae possesses a red pigment which is concentrated anteriorly around the pharynx and brain and has a diffuse distribution throughout the rest of the body, apart from a slight concentration at the extreme posterior. The pigment was identified as a haemoglobin by its positive reaction to the cyanol blue and diaminobenzidine techniques (Fig. 1). These reactions were obtained in living specimens and others fixed in 10% neutral formalin; pre-heating specimens to 90° C for up to 1 hr did not abolish the reaction, demonstrating that it was not caused by peroxidases or catalases. Exposure of living specimens to carbon monoxide by gently bubbling the gas through the sea water changed the normal orange to red coloration to a permanent, bright, cherry-red but this did not affect the *in vitro* survival rate, treated specimens surviving as long as untreated ones away from the host.

The pigment is absent from the embryos but appears to develop soon after birth, judging from its presence in small immature adults which lack, as yet, any trace of the reproductive system.

In contrast, no reactions for haemoglobin could be obtained in either of the other two species studied.

Paravortex cardii

88.2% of the *Cerastoderma edule* from Plymouth contained *Paravortex cardii;* the overall mean infection rate was 5.7 rhabdocoels per host with a range of 0 to 38 and a variance of 0.03. It was found that 62.5% of those from Spurn Head were infected, with an overall mean infection rate of 1.9 per host, a range of 0 to 26 and a variance of 0.27. Both populations of rhabdocoels resembled that of *P. scrobiculariae* in showing no seasonal fluctuations; *P. cardii,* like *P. scrobiculariae,* is viviparous, and adults containing embryos were found throughout the year.

Diet, feeding, gut structure, digestion and food reserves. The general pattern of nutrition in *P. cardii,* both intrinsically and in relation to its bivalve host, is virtually identical with that described for *P. scrobiculariae* and will not, therefore, be considered under separate headings. The only major difference is that *P. cardii* does not show any migration within the gut of its host for feeding purposes but lives permanently in the main ducts of the digestive gland. Previous accounts (*e.g.*, Hallez, 1909; Atkins, 1934) state that *P. cardii* occurs in the stomach of *Cerastoderma edule,* but careful dissection of bivalves killed instantaneously by plunging them into *iso*-pentane cooled in liquid nitrogen shows that the rhabdocoels are, in fact, always located within the main ducts of the digestive gland irrespective of tidal or other factors. Dissection or fixation of living bivalves causes contractions throughout the body which expel many of the rhabdocoels into the stomach and this is presumably the reason for the earlier erroneous reports. Rhabdocoels were only rarely found in the secondary ducts of the digestive gland, however, and this is believed to be due to the small size of the ducts relative to those of *Scrobicularia plana,* which can be up to two or three times wider.

Our observations on feeding and digestion in *C. edule* agree broadly with those by Owen (1972) on this bivalve and hence will be reported only briefly. The specimens studied came from near low water mark and were usually submerged, therefore, for a much larger proportion of each tidal cycle than *S. plana.* Consequently, feeding and digestion were found to be virtually continuous and they did not show the cyclical features seen in the latter species.

Specimens fixed on the shore usually had full stomachs in which diatoms, other unicellular algae and protozoa predominated. Organic detritus, so abundant in the stomach of *S. plana,* was present in much smaller amounts, but both this and the food organisms contained small but demonstrable quantities of iron, which again formed a convenient marker of subsequent digestive processes. It was apparent from these specimens, and others in laboratory simulations, that the more or less continuous feeding is accompanied by extracellular digestion in the stomach and movement of both intact and semi-digested food into the digestive gland. Here uptake of food by the digestive cells and the completion of digestion intracellularly occur by processes very similar to those seen in *S. plana.* The range of enzymes demonstrated in the stomach contents and primary lysosomes of the digestive cells was the same as in that species and a few iron-rich residual bodies were again visible endproducts. The digestive cells, however, take up materials in different amounts and at different rates; synchronous stages within tubules or sets of tubules were never seen and the residual bodies are usually eliminated by abstriction. Cells which have voided their residual bodies appear to regenerate; only rarely was complete degeneration and shedding of digestive cells seen.

P. cardii ingests the intact and partly digested components of the host's food as they pass through the main ducts into the digestive gland. Unaltered mucus, from either the original food-string of the host or the stomach, was often present in the ducts and this, too, was ingested by the rhabdocoel. Residual bodies, some clearly "labeled" with inorganic iron, from the digestive gland are also ingested as they pass down the ducts to the stomach.

The gut structure is very similar to that of *P. scrobiculariae,* except that the gastrodermal cells are somewhat smaller, measuring 50 to 60 μm by 15 to 20 μm, and do not tend to occlude the intestinal lumen. Starved specimens showed slight to moderate amounts of non-specific esterases, A- and C-esterases, acid and alkaline phosphatases, lipase and β-glucuronidase in their gastrodermal cells. As in the case

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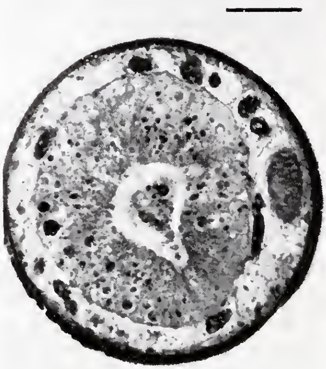

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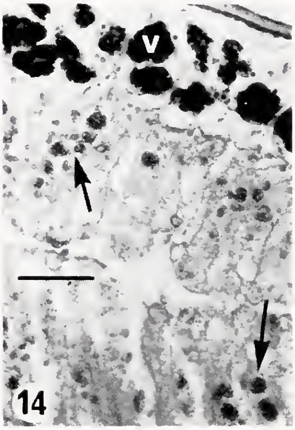

FIGURE 12. *P. cardii.* A whole mount preparation of an individual fixed immediately after removal from a main duct of the digestive gland of a naturally fed *Cerastoderma edule.* The entire intestine shows an intense reaction for β-galactosidase; in the original preparation this can be seen to be localized in the contents of both the gut lumen and the intracellular vacuoles of the gastrodermis. The preparation was treated by the method for β-galactosidase described by Pearse (1972); the scale is 0.3 mm.

FIGURE 13. *Graffilla buccinicola.* A transverse section through the middle region of a mature individual dissected from the digestive gland of *Buccinum undatum.* The intestinal lumen contains cellular debris derived from the host's digestive epithelium and the gastrodermal cells show many heterogeneous inclusions resulting from phagocytosis of such debris. The section is stained by Mallory's trichrome method; the scale is 0.1 mm.

FIGURE 14. *G. buccinicola.* Part of a longitudinal section of an individual fixed immediately after removal from the digestive gland of *B. undatum* and showing a portion of the gastrodermis, the intestinal lumen, some vitellaria (v) and the epidermis. The intracellular inclusions (arrows) and vitellaria show medium and strong reactions, respectively, for β-glucuronidase. The section was treated for demonstration of β-glucuronidase by the method of Pearse (1972); the scale is 0.05 mm.

of *P. scrobiculariae*, however, dramatic increases occurred in the intensity of the reactions for these enzymes after *P. cardii* had fed *in situ* within its host and some individuals showed, additionally, strong positive reactions for β-galactosidase (Fig. 12). Specimens fed *in vitro* on enzymically inert foods did not develop such reactions and it is concluded that in this species, also, most of the enzymes responsible for digestion are derived from the host.

The food reserves resemble those of *P. scrobiculariae* and consist mainly of glycogen, supplemented by small amounts of lipid, laid down in the gastrodermis and mesenchyme.

Graffilla buccinicola

82.4% of the *Buccinum undatum* examined contained *Graffilla buccinicola;* the overall mean infection rate was 5.9 rhabdocoels per host with a range of 0 to 18 and a variance of 0.029. The rhabdocoel is hermaphrodite but not viviparous and specimens from both *B. undatum* and *Neptunea decemcostata* showed that the species is protandrous with considerable hypertrophy of the vitellaria during the female phase. No significant differences in diet or gut structure were found in specimens from the two hosts; no observations on digestive enzymes could be made on those from *N. decemcostata,* however, as only Bouin-fixed material was available.

Structure of the gut. The gut is of the usual dalyellioid form but differs from that of the two species of *Paravortex* in that the pharynx is larger, with very well-developed musculature, and the gastrodermal cells lack the large intracellular fluid-filled vacuoles so characteristic of both *P. scrobiculariae* and *P. cardii.* The cells are monotypic, columnar, 75 to 100 μm tall and 30 to 50 μm wide, with basal nuclei and cytoplasm usually containing heterogeneous inclusions ranging in diameter from 2 to 20 μm (Fig. 13). These inclusions lie in vacuoles; their staining reactions vary enormously and they are very similar to those seen in cells of the hosts' digestive glands.

The food and feeding behavior. G. buccinicola lives permanently in the ducts and tubules of the digestive gland of *B. undatum* and was never found at other sites despite earlier reports (*e.g.,* Jameson, 1897; Dakin, 1912; Westblad, 1926) of its occurrence in the kidney and kidney ducts. Only the digestive gland of *N. decemcostata* was available for examination; the rhabdocoel was found in the ducts and tubules but Tomkiewicz and Morse (personal communication) report it also from the stomach of this host.

Feeding and digestion in the host B. undatum. B. undatum has carnivorous and scavenging habits and these are reflected in its digestive physiology, which is of the general pattern for carnivorous gastropods as reviewed by Owen (1966) and Purchon (1977). Gastric digestion is extensive and is followed by movement of soluble, largely proteinaceous, materials into the digestive gland. Here they are taken up by the cells of the tubules and digestion is completed intracellularly by non-specific esterases, A- and C-esterases, arylamidases, lipase and β-glucuronidase in a lysosomal sequence similar to that described for *S. plana* and *C. edule.* Acid phosphatase is present in the primary lysosomes, also, and the arylamidases occur in very large amounts. The heterolysosomes in which intracellular digestion proceeds have a great affinity for both acidic and basic stains and the residual bodies are usually rich in iron and calcium, as in *S. plana.* The digestive cells and tubules do not show cyclic activity, possibly because of the gastropod's irregular feeding habits; the residual bodies are eliminated by abstriction or, rarely, by cell disintegration and all stages of intracellular digestion can usually be found in adjacent tubules.

Diet and feeding in G. buccinicola. The rhabdocoel feeds in part on the host's semidigested food, as this passes into the digestive gland, and on discarded residual bodies. There are strong indications that it also takes in digestive cells from the tubule epithelium. The intestinal lumen often contains much cellular debris (Fig. 13) which is clearly derived from host digestive cells; the discrepancy between the large amounts regularly present and the observed low rate of disintegration of digestive cells suggests that the rhabdocoel is actively removing cells from the epithelium. This is supported by the presence in the intestinal lumen, and also intracellularly in the gastrodermis, of large host heterolysosomes still containing much protein and manifestly not yet in a condition suitable for discarding from their formative cells. Further support came from the observation of damaged areas of tubule epithelium; these consisted of gaps three to four cells wide and in no way resembled the areas left by the normal shedding of cells seen in the bivalves studied.

G. buccinicola penetrates much farther into the host's digestive gland than do either *P. scrobiculariae* or *P. cardii* and is sometimes found within a digestive tubule pressed against the epithelium or, more rarely, outside the tubule proper but lying between adjacent tubules. This behavior is in accord with the postulated feeding on intact digestive cells and might account for the earlier reports of specimens from other sites in the body.

Digestion and food reserves. The gastrodermis of specimens maintained *in vitro* for up to 4 days showed only low levels of enzymic activity. The enzymes demonstrated were non-specific esterases, A- and C-esterases, lipase, arylamidases, β-glucuronidase, and acid and alkaline phosphatases. In contrast, others fixed directly on removal from the host showed very high levels of activity for all these enzymes, in both the lumen contents and the intracellular inclusions (Fig. 14). Unfortunately, specimens kept *in vitro* refused to feed on any type of enzymically inert food, so that it was impossible to determine whether or not this increased activity was endogenous or, as in *P. scrobiculariae* and *P. cardii,* of host origin. The latter seemed to be the more likely, though, taking into account the high level of activity in the lumen contents and the absence of any glandular components from the gastrodermis. Of the various enzymes demonstrated, arylamidases were particularly active; this was also the case in the host digestive cells, lending more support to the case for involvement of host enzymes in the rhabdocoel's digestive processes.

The food reserves resemble those of *P. scrobiculariae* and *P. cardii,* lipid being deposited sparingly in the gastrodermis and in some abundance in the vitellaria. Glycogen is abundant in the mesenchyme and especially so in the vitellaria. The latter also show intense reactions for non-specific esterases, lipase, acid and alkaline phosphatases and β-glucuronidase (Fig. 14) and are clearly sites of sustained metabolic activity.

Discussion

The salient point emerging from these observations is that the three entosymbiotic rhabdocoels studied show adaptive features in their nutritional physiology which indicate a definite trend toward parasitic habits.

The basic nutritional pattern in all three species would seem to be derived from a commensal type, since a proportion of their diet consists of the hosts' food; they do not, however, abstract this until it has undergone preliminary digestion by the hosts' gastric enzymes, and these continue to act within the flatworms' gut. This extension of a commensal habit to include utilization of the host's digestive enzymes could logically be expected to result in decreased production of enzymes within the symbiotes' own alimentary systems and to culminate, perhaps, in the loss of all endogenous digestive enzymes. The initial stages of this process are, in fact, found in all three species; gland cells producing extracellularly-acting proteases which are a common feature of many turbellarian alimentary systems (Jennings, 1974b) are absent and enzyme production in the monotypic gastrodermal cells is at a consistently low level. This contrasts sharply with the situation in rhabdocoels symbiotic with echinoderms, where the food consists of either co-symbiotes or the host's unaltered food and where the digestive physiology is very similar to that of free-living species (Jennings and Mettrick, 1968; Mettrick and Jennings, 1969; Jennings, 1977).

Since the rhabdocoels' digestive enzymes are supplemented by those of their hosts, it is not surprising that the types identified in their gut cells are appropriate to the diet of the particular host. *Graffilla buccinicola,* for example, from a carnivorous host, shows high levels of arylamidase activity during digestion as compared with the two species of *Paravortex* which come from omnivorous, largely herbivorous, hosts.

Utilization of the hosts' digestive enzymes has probably been facilitated by the peculiarities of the molluscan digestive system. The persistence in both bivalves and gastropods of extensive intracellular digestion within a specialized region of the gut, the digestive gland, provides a suitable feeding site for entosymbiotes away from the variable conditions of the stomach, but where products of gastric digestion, and gastric enzymes, are still available. Also, the elimination of unwanted end-products (spent or partially spent heterolysosomes) by either cell disintegration or abstriction provides a further source of both food and enzymes for the rhabdocoels. The digestive gland enzymes released during this elimination are believed to be important in the hosts' gastric physiology (Morton, 1956; 1967; Purchon, 1977) and so it is not surprising that they, too, are utilized by the symbiotes.

Use of cellular debris as food was probably the initial stimulus for the extension of the diet, in *Graffilla buccinicola,* to include intact host digestive cells. Here, the hyper-development of the pharynx relative to those of the two species of *Paravortex* is no doubt significant and *G. buccinicola* seems to be approachng the status of a tissue-feeding parasite of the type exemplified by some adult digenetic trematodes (Halton, 1967).

The iron contained in the residual bodies shed from the hosts' digestive glands and subsequently ingested by the rhabdocoels appears to be eliminated from the symbiotes' gastrodermis in soluble form, probably via the excretory system, and not by cell disintegration or abstriction. The ability to deal with the metal in this way is possibly another adaptation to entosymbiosis in molluscs and the particular types of food made available by this mode of life; the presence of iron in relatively large quantities was a consistent feature of the digestive glands of all four host species studied and may well be a characteristic of many other molluscs. It has been reported, for example, from the digestive glands of the bivalves *Nucula sulcata* (Owen, 1973) and *Mytilus edulis* (M. N. Moore, personal communication), and from populations of *Scrobicularia plana* widely separated, geographically, from those sampled in the present study (Bryan and Hummerstone, 1978; Bryan and Uysall, 1978).

Elimination of dietary iron in soluble form via the excretory system in the manner suggested for these entosymbiotic rhabdocoels is believed to occur also in certain sanguivorous digenetic trematodes (Jennings, 1968). This common ability to deal with excess dietary iron without cell disintegration, and the fact that the molluscan digestive gland remains the favored habitat of larval Digenea, lend further support to the hypotheses that the Digenea arose from rhabdocoel-like ancestors and that the digestive gland is the primitive habitat of rhabdocoels living entosymbiotically in molluscs. Sanguivorous Monogenea, in contrast, do eliminate excess iron by cell disintegration (Halton and Jennings, 1965) ; these are typically ectoparasites of fishes and have no molluscan connections in their life cycles.

If the digestive gland is the primitive habitat, then the interesting migrations of *Paravortex scrobiculariae* between the gland and the intestine seem to be somewhat anomalous. The reasons for the migrations remain unknown, although they are presumably related in some way to the sharply defined feeding and digestive cycles of the particular host and hence, indirectly, to the tidal cycle. It may be that the ducts of the digestive gland are untenable at certain points in the host's digestive cycle, although, over the period in which the rhabdocoels are in the intestine (approximately mid-flood tide to mid-ebb tide), conditions would seem to be optimal for a stay in the gland. In the middle of this period, at high water, the host is feeding actively, food is passing into the stomach and beginning to enter the digestive gland and there should be maximum amounts of oxygen available, as considerable quantities of water are passing through the bivalve's filtration system. It is during the opposing phase, though, that *P. scrobiculariae* is to be found in the gland and, while the supply of partially digested food and cell debris is now maximal, the host's filtration system has closed down and oxygen is likely to be in short supply. In this situation, however, the adaptive significance of the haemoglobin present in this species becomes apparent. The pigment has been shown by spectrographic methods to be physiologically active and capable of reversible reactions with oxygen depending on the environmental oxygen tension (Phillips, 1978). Its concentration around the brain and pharynx suggests that it may well be important in supplying oxygen to these structures during feeding when the host is not filtering sea water and the oxygen tension of the mud habitat is very much reduced. The haemoglobin is obviously not essential for life under normal aerobic conditions, at least *in vitro,* specimens treated with carbon monoxide surviving for as long as untreated specimens. The carbon monoxide formed an apparently stable compound with the haemoglobin, judging from the permanence of the cherry red coloration such treatment induces, and this test is generally regarded as indicating whether or not an animal's haemoglobin is essential for survival (Prosser, 1973). The small amount of haemoglobin present would not seem to be capable of acting as an oxygen store for use over the period of feeding at low water, but it may be adequate for a process of facilitated diffusion of the type postulated by Wittenberg (1970). This would allow the rhabdocoel preferential use of such oxygen as is available, its host apparently lacking either haemoglobin or haemocyanin in common with most other bivalves (Morton, 1967). It would be interesting to attempt the carbon monoxide treatment *in vivo* in the host's natural habitat to test this hypothesis.

Paravortex cardii and *Graffilla buccinicola,* living in hosts whose habitats are permanently well-aerated and not subject to changes in oxygen tension, understandably lack haemoglobin.

The occurrence of haemoglobin in *P. scrobiculariae* is not unique in the Rhabdocoela. It has been reported, for example, in *Phaenocora unipunctata* and

P. typhlops (Crompton and Smith, 1963; Young and Harris, 1973) and both these species, significantly, are mud-dwellers living in conditions of low or variable oxygen tension. Another mud-dweller, the nematode *Enoplus brevis,* has a pharyngeal haemoglobin which presumably facilitates feeding, while its close relative *E. communis,* which lives in more aerated situations, does not (Atkinson, 1977).

The food reserves of all three species with their emphasis on glycogen storage, and the reproductive strategies of the two species of *Paravortex* with their emphasis on production of large numbers of offspring throughout the year, both conform to the pattern described for other entosymbiotic Platyhelminthes by Calow and Jennings (1974) and Jennings and Calow (1975). Very little information is available on the reproductive strategy of *G. buccinicola,* but the hypertrophy of its vitellaria, relative to those of free-living rhabdocoels, implies a similar emphasis on reproduction.

The peculiar supplementation of the nutrition of the embryo in the viviparous *P. scrobiculariae* and *P. cardii* by transfer of nutrients, sometimes particulate and undigested, from the parental gut is possibly a further adaptation to entosymbiosis. It may well enhance the chances of survival of the juveniles by allowing them to develop as far as possible, and certainly to a point where they can feed in the adult manner, before they leave the parent. Remarkable though this method of embryonic nutrition is, if it is accepted that entry of eggs or embryos into the gut for subsequent passage to the exterior via the mouth is an archaic turbellarian feature (*vide* Hyman, 1951), there is no reason why the embryos should not ingest gut contents during the process. On this view, transfer of nutrients to embryos still within the mesenchyme is simply an extension of the same phenomenon. There is, in fact, a precedent for unusual modes of embryonic nutrition in the genus *Paravortex;* Ball (1916) described uptake of free yolk globules by wandering amoeboid ectoderm and primary entoderm cells in gastrulae and post-gastrulae of *P. gemellipara,* another viviparous species which is entosymbiotic in the bivalve *Modiolus,* and stated that the laden entoderm cells are then engulfed by the secondary entoderm. Ball does not mention any features in the nutrition of the advanced embryo comparable to those reported here, but the techniques used in his primarily developmental study would most probably have not revealed them.

SUMMARY

1. Diet, gut structure, digestive physiology and food reserves have been studied in three entosymbiotic graffillid rhabdocoels.

2. In all three species these aspects of their nutritional physiology are much modified relative to those characteristic of free-living flatworms; they show adaptive features related to the entosymbiotic habit and, in particular, to the feeding and digestive processes of the respective hosts.

3. The three species feed on their hosts' partially digested food and the cellular debris released at the end of the digestive cycle occurring within the hosts' digestive gland. In one species this is extended to include removal of intact cells from the digestive epithelium.

4. The ingested food contains enzymes of host origin which continue to act within the rhabdocoels' alimentary systems and play a dominant role in the digestive physiology. There is concomitant reduction in the amounts of enzymes produced by the symbiotes.

5. Food reserves are of the type and relative amounts characteristic of other entosymbiotic Platyhelminthes and consist mainly of glycogen.

6. Two of the species studied are viviparous and there is evidence that advanced embryos receive nutrients from the parental gut.

7. One species, which migrates from the host's intestine into the digestive gland to feed during the mid-ebb to mid-flood tidal period, possesses an endogenous haemoglobin. It is suggested that this helps overcome oxygen deficiencies, during feeding, by facilitated diffusion. The other two species, which live and feed in conditions of more constant oxygen availability, lack haemoglobin.

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COLCHICINE AND CYTOCHALASIN B: A FURTHER CHARACTERIZATION OF THEIR ACTIONS ON CRUSTACEAN CHROMATOPHORES USING THE IONOPHORE A23187 AND THIOL REAGENTS

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Among the various examples of intracellular movement, one of the more easily studied is the migration of pigment granules within the cytoplasm of chromatophores. By controlling the distribution of the pigment within these cells, an animal possessing chromatophores is able to adjust its coloration to suit such purposes as camouflage (Sumner, 1935), temperature regulation (Wilkens and Fingerman, 1965), or protection from ultraviolet light (Coohill, Bartell, and Fingerman, 1970). When the pigment of a given chromatophore is dispersed throughout the cytoplasm, thereby covering a greater surface area, the tissue bearing the chromatophore takes on the tint of the latter. Alternatively, when the pigment is concentrated into the center of the cell, it contributes little to the coloration of the tissue.

As is the case for many other forms of intracellular movement, several studies have implicated microtubules and/or microfilaments as the agents responsible for translocation of pigment granules in chromatophores of vertebrates, crustaceans, and echinoderms. These studies have frequently employed colchicine, which has long been held to selectively disrupt microtubules (Borisy and Taylor, 1967a, b; Shelanski and Taylor, 1967) and cytochalasin B (CB), which has been promoted as a probe for microfilament involvement in cell functions by Wessells, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn, and Yamada (1971).

Reports on CB action on chromatophores have been variable. The drug inhibits both pigment dispersion and concentration in crustacean and echinoderm chromatophores (Lambert and Crowe, 1973, 1976; Robison and Charlton, 1973; Fingerman, Fingerman, and Lambert, 1975; Dambach and Weber, 1975), enhances both dispersion and concentration in fish melanophores (Ohta, 1974), and inhibits pigment dispersion in frog melanophores while having variable effects on pigment concentration (Malawista, 1971b; McGuire and Moellmann, 1972; Novales and Novales, 1972; Magun, 1973; Fisher and Lyerla, 1974).

Colchicine has been found to inhibit pigment concentration in all chromatophores studied, in both vertebrates (Wright, 1955; Malawista, 1965, 1971a; Wikswo and Novales, 1969, 1972a; Schliwa and Bereiter-Hahn, 1973; Murphy and Tilney, 1974) and crustaceans (Lambert and Crowe, 1973, 1976; Fingerman *et al.*, 1975). The reported effects of colchicine on pigment dispersion are more variable. In frog melanophores the drug enhances the action of melanotropin (melanocyte-stimulating hormone, MSH) in bringing about pigment dispersion, and also causes dispersion itself (Malawista, 1965; Fisher and Lyerla, 1974). A similar situation has been reported for the teleost, *Fundulus heteroclitus* (Wikswo and Novales, 1969). In contrast, colchicine inhibits hormone-induced dispersion in erythrophores of at least one species of crustacean (Fingerman *et al.*, 1975) and does not itself cause pigment dispersion in melanophores of another (Lambert and Crowe, 1976). It is clear that, so far as pigment dispersion is concerned, colchicine's action on crustacean chromatophores differs markedly from its action on those of vertebrates. In contrast, pigment concentration by both types of chromatophore is inhibited by the drug. Wikswo and Novales (1972b) have suggested that colchicine exerts its inhibitory action by reacting with thiol groups, presumably of microtubules. To determine whether colchicine inhibits pigment concentration in crustaceans in the same manner in which it acts on vertebrates, the interactions of thiol reagents with the melanophores of *Uca pugilator* are examined in the first part of this paper.

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That neither colchicine nor CB is as specific as once thought is now well established. For instance, both colchicine and CB have been found to inhibit a variety of plasma membrane transport systems (Mizel and Wilson, 1972a, b; Bos and Emmelot, 1974) and CB has even been found to bind with high affinity to the hexose transport proteins of the erythrocyte membrane (Lin and Spudich, 1974a, b). One of colchicine's non-microtubular actions appears to be interference with the mobility of intramembranous particles (Wunderlich, Müller, and Speth, 1973; Furcht and Scott, 1975). On the basis of experiments in which lumicolchicine, an isomer of colchicine which does not bind tubulin, was as effective as colchicine in inhibiting pigment concentration, Lambert and Fingerman (1976) suggested that: first, the inhibition of pigment concentration by colchicine was not due to microtubular disruption; and second, that colchicine might act at the level of the plasma membrane, perhaps interfering with the behavior of the hormone-receptor complex. Considering the diverse effects of CB in various systems, it was suggested that this drug might also act at the plasma membrane level (Fingerman *et al.*, 1975).

One way to distinguish whether or not these drugs act at the level of the hormone receptor, rather than on the pigment-moving machinery itself, is to examine their effects on pigment movement when the hormone-receptor system is bypassed. Such a bypass system has recently been devised, utilizing the calcium ionophore A23187 (Lambert and Fingerman, 1977). This ionophore has served as a useful tool for investigating the role of Ca^{++} in a variety of physiological processes, acting by shuttling Ca^{++} (or other divalent cations) across a biological membrane down the cation's concentration gradient (Pressman, 1976). When applied to erythrophores of the prawn, *Palaemonetes pugio,* the ionophore appears to mimic the action of the red pigment-concentrating hormone (RPCH), both agents presumably causing an influx of Ca^{++} which somehow activates the pigment-concentrating mechanism in the erythrophores (Lambert and Fingerman, 1977). The ionophore has also been recently applied to amphibian melanophores, where in contrast to the prawn erythrophores, pigment dispersion is induced (van de Veerdonk, Worm, Seldenrijk, and Heussen, 1977; Novales, 1977). In the second part of this paper, the effects of colchicine and CB on ionophore-induced pigment translocation are described.

MATERIALS AND METHODS

Two crustacean species were used in this study; the fiddler crab, *Uca pugilator,* obtained commercially from the Gulf Specimen Company, Panacea, Florida, and the prawn, *Palaemonetes pugio,* collected near Irish Bayou, Louisiana. With the fiddler crab, the chromatophores studied were the melanophores on the merus of the third and fourth walking legs, using the perfusion technique of Fingerman (1956). This involved removing a pair of legs from each crab, inserting a 26 gauge needle into the distal end of each leg, and perfusing 0.1 ml of a given solution through the leg. One member of the pair would receive the test substance (*e.g.*, colchicine), the other a control solution (*e.g.*, physiological saline). Following perfusion, each leg was placed in a 0.1 ml drop of the same type of solution that had been perfused through it. This drop was placed in a petri dish whose air was saturated with water by the inclusion of pieces of filter paper soaked in distilled water. The saline used for the crab experiments was that of Pantin (1934). For experiments with the prawn, the large dark erythrophores of the ovaries were the chromatophores studied. The ovaries were removed and hemisected, one half being incubated in a control solution, the other half in the experimental solution. The physiological saline for these experiments was that of van Harreveld (1936). For both species, the responses of the chromatophores were recorded according to the scheme of Hogben and Slome (1931), in which stage 1 represents maximal pigment concentration, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions.

Of the substances tested, colchicine, cysteine hydrochloride and mersalyl acid were obtained from Sigma and glutathione was purchased from Calbiochem. CB (Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and stored at 4° C as a stock solution of 1 mg/ml. The ionophore A23187, a gift from Dr. R. Hamill, Eli Lilly Co., was also dissolved in DMSO, to give a 5 mM stock solution which was stored at 4° C in the dark.

Preparation of melanophores of *Uca pugilator* for electron microscopy involved fixation in 6% glutaraldehyde in 0.1 M cacodylate buffer, osmolarity adjusted to 1100 with NaCl, the fixative being delievered to the cells by perfusing it through isolated legs on ice for 45 min. Post-fixation with OsO_4 was omitted to avoid its degrading action on actin filaments. Following fixation, the tissue was dehydrated through an acetone series, embedded in Spurr's (1969) epoxy resin, and sectioned with a Porter-Blum MT-2 microtome. Sections were collected on formvar-coated grids, then stained with 4% methanolic uranyl acetate at 65° C for 15 min, followed with lead citrate. The stained sections were examined with a Siemens Elmiskop 1A transmission electron microscope at an accelerating voltage of 80 *k*V.

RESULTS

Sulfhydryls

The suggestion by Wikswo and Novales (1972b) that colchicine exerts its inhibitory action by reacting with thiol groups resulted from their studies with,

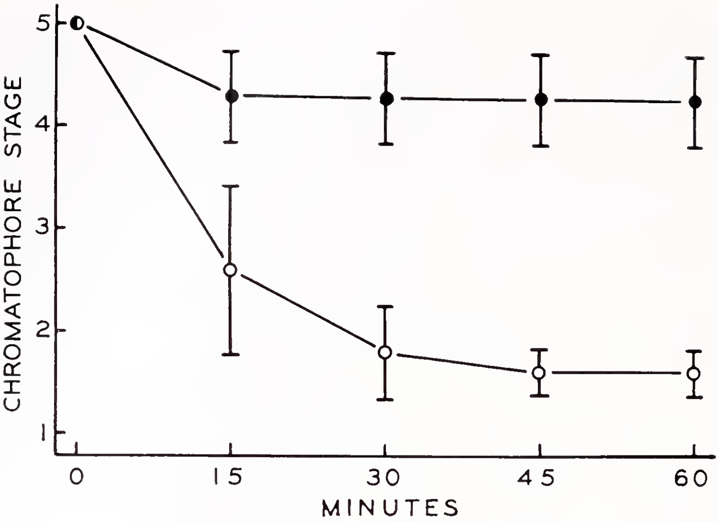

FIGURE 1. Effect of mersalyl acid on pigment concentration in leg melanophores of *Uca pugilator*. Opposite legs were removed; the experimentals (dots) were perfused wth saline containing 37 mM mersalyl acid, the controls (circles) with saline alone. Five pairs were used. Vertical lines representing one standard deviation on each side of the mean are included in this and the following graphs.

among other reagents, glutathione, cysteine hydrochloride, and mersalyl acid. To further characterize the site of action of colchicine in crustacean chromatophores, these reagents were applied to the melanophores of *Uca*, using the isolated leg-perfusion technique. As has been described previously, perfusing legs with saline brings about pigment concentration; when high concentrations of colchicine are included in the saline, this pigment movement is inhibited (Lambert and Crowe, 1973). To determine whether glutathione or cysteine hydrochloride could prevent colchicine's inhibition of pigment concentration, as was reported for the melanophores of *Fundulus* (Wikswo and Novales, 1972b), these reagents were mixed with colchicine and perfused through isolated legs. The control legs were perfused with colchicine alone. At the three concentrations used (50, 100, and 155 mM), glutathione was unable to prevent inhibition by 15 mM colchicine (data not shown). Similarly, 210 mM cysteine hydrochloride did not counteract inhibition by 21 mM colchicine (data not shown). In contrast to the differences between the melanophores of *Uca* and *Fundulus* described above, pigment concentration is inhibited in the melanophores of both upon treatment with mersalyl acid. As seen in Figure 1, perfusing mersalyl acid (37 mM) through isolated legs greatly inhibits pigment concentration in the melanophores of these legs, as compared to the controls perfused with saline alone. Lower concentrations of mersalyl acid (10 mM) were ineffective (data not shown). In contrast to colchicine's action, the inhibitory potential of mersalyl is negated by mixing it with 155 mM glutathione before perfusing it through the legs (Fig. 2).

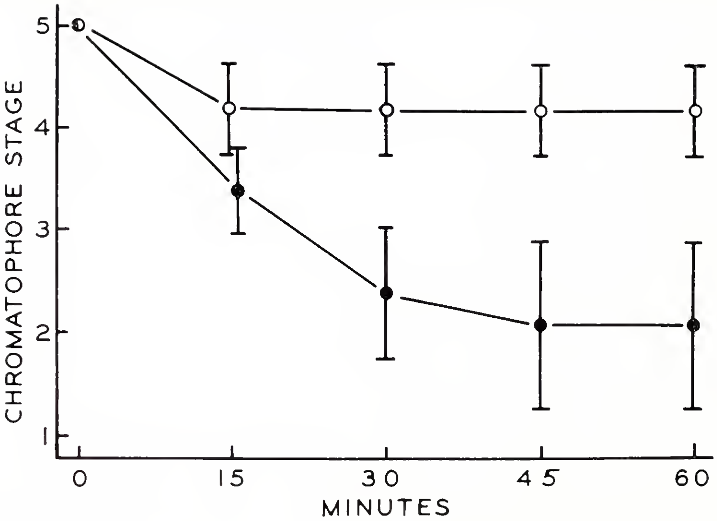

FIGURE 2. Effect of glutathione on mersalyl acid inhibition of pigment concentration in leg melanophores of *Uca pugilator*. Opposite legs were removed; the experimentals (dots) were perfused with saline containing 37 mM mersalyl acid plus 155 mM glutathione, the controls (circles) with saline containing 37 mM mersalyl acid alone. Five pairs were used.

A23187/colchicine effects

To determine whether colchicine acts at the hormone-receptor level or at some more distal site (*e.g.*, microtubules), its ability to prevent ionophore-induced pigment movements was examined, using the ovarian erythrophores of *Palaemonetes pugio*. As seen in Figure 3, I, erythrophores treated with 25 mM colchicine responded as well to the ionophore as untreated cells, even after a 30 min preincubation in the colchicine. The presumed influx of Ca^{++} from the medium into the cells caused rapid pigment concentration in both cases. In contrast, colchicine appears to inhibit the pigment dispersion that normally occurs when A23187-treated erythrophores are exposed to Ca^{++}-free/EGTA-containing saline (Fig. 3, II). The erythrophores on four of the five control ovary halves used (*i.e.*, those not treated with colchicine) dispersed their pigment, presumably due to the efflux of Ca^{++}, while all of the colchicine-treated erythrophores remained in a concentrated condition. The degree of re-dispersion was variable among the controls, probably due to damage to the erythrophores when the ovaries were bisected. Ionophore-treated cells appear to respond to Ca^{++}-free/EGTA saline best when treated very gently.

Cytochalasin B (CB) and A23187

To characterize further the inhibitory action of cytochalasin B on pigment concentration in crustacean chromatophores, its effect on A23187-induced pigment concentration was studied using ovarian erythrophores of *Palaemonetes pugio*.

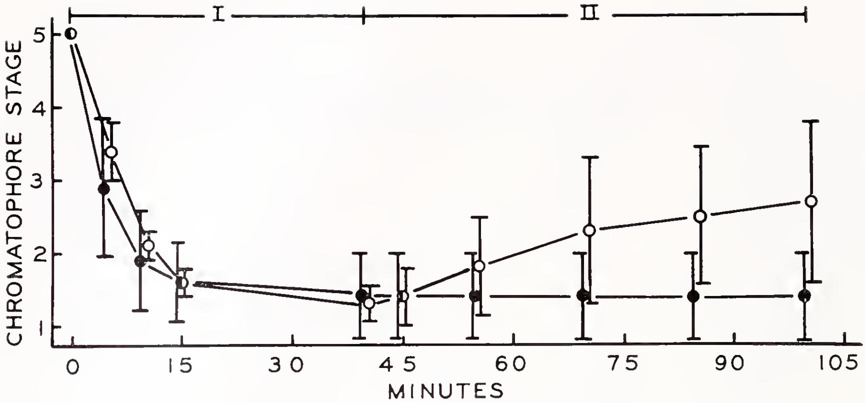

FIGURE 3. Effect of colchicine on ionophore A23187-mediated pigment movement in ovarian erythrophores of *Palaemonetes pugio*. Ovaries were removed and bisected, the experimental halves (dots) being pre-incubated in saline containing 25 mM colchicine and the controls (circles) in saline alone. After 30 min (at t = 0 min), the ionophore was added to all incubation media to a concentration of 25 μM, starting phase I. Forty minutes later, the test solutions were replaced with Ca^{++}-free/5 mM EGTA saline containing (dots) or lacking (circles) 25 mM colchicine, starting phase II. Five ovaries were used.

As seen in Figure 4, those erythrophores treated with CB respond less well than untreated cells to ionophore-induced pigment concentration. This inhibition is, however, partially reversed by the removal of CB and addition of fresh ionophore.

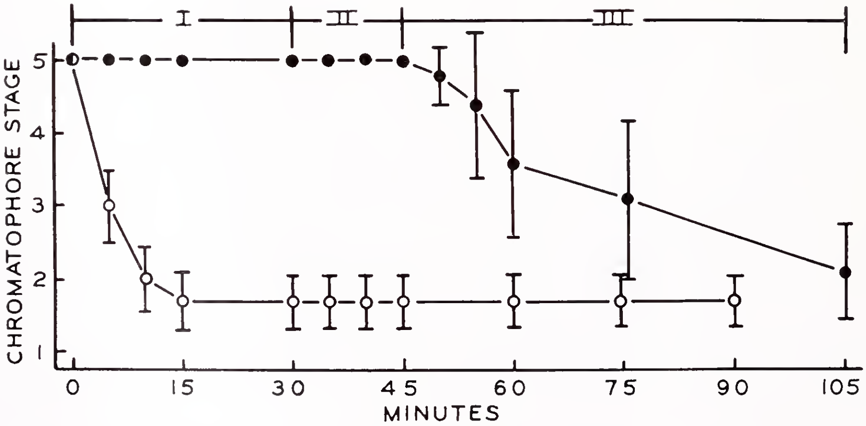

FIGURE 4. Effect of cytochalasin B on ionophore A23187-induced pigment concentration in ovarian erythrophores of *P. pugio*. Ovaries were removed and bisected, the experimental halves (dots) then being pre-incubated in 0.2 ml saline containing 1% DMSO and 10 μg/ml CB and the controls (circles) in saline containing 1% DMSO alone. After 20 min pre-incubation (at t = 0 min), the ionophore was added to all test solutions to a concentration of 25 μM, initiating phase I. At the start of phase II, all ovary halves were washed with fresh saline. At the start of phase III, this saline was replaced with fresh saline containing 25 μM A23187 and 0.5% DMSO. Ten ovaries were used.

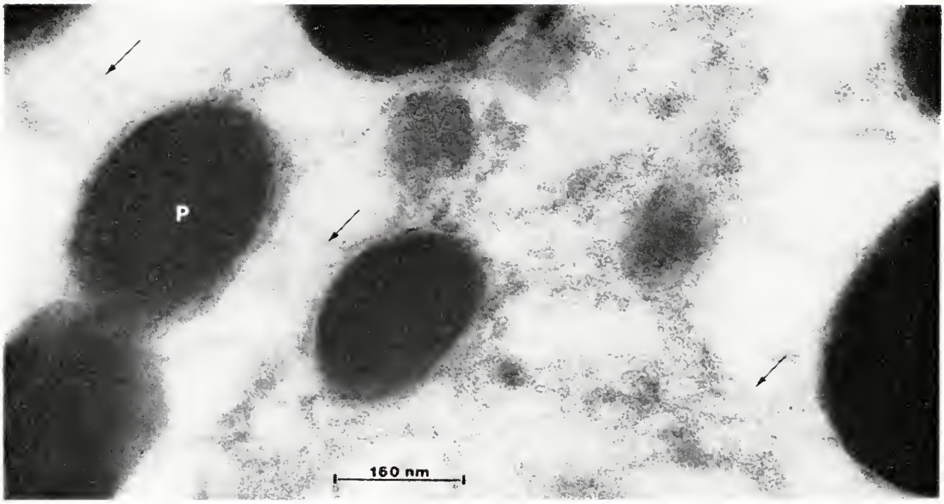

FIGURE 5. Electronmicrograph of a melanophore process from *Uca*. Several pigment granules (P) are seen in this portion of a process. They appear to be interconnected by the elements of the filamentous matrix (arrows). Bar represents 160 nm.

Electron microscopy

Earlier attempts at finding actin-like filaments in crab melanophores through the use of electron microscopy have been unsuccessful (Lambert, 1975). A possible explanation for this was recently provided by the observation of Maupin-Szamier and Pollard (1978) that OsO_4 degrades purified actin filaments. Since OsO_4 was included in the earlier fixation procedures for the crab melanophores, it was decided to try another fixation using only glutaraldehyde. Using the fixation procedure described under Materials and Methods, the cytoplasm of the melanophores of *Uca pugilator* was seen to consist of irregular filamentous elements which appear to contact, and interconnect, the pigment granules (Fig. 5). The dimensions of these elements are variable, ranging from 2.5 to 25 nm. In addition, they appear to coalesce frequently, both with each other and with more amorphous aspects of the matrix.

DISCUSSION

The attempt of this study to characterize further the modes of action of colchicine and CB on crustacean chromatophores has provided further evidence that microtubules are not necessary for pigment concentration, though a role in dispersion remains a possibility. The site of action of CB remains unclear.

A number of lines of evidence now indicate that colchicine's inhibitory action on pigment concentration in crustacean chromatophores is not mediated by microtubular disruption. One is the observation by Fingerman *et al.*, (1975) that very high doses of colchicine (25 mM) are required to inhibit pigment concentration in erythrophores of *Palaemonetes vulgaris,* while much lower doses (1 mM) are sufficient for microtubular disruption in these cells (Robison and Charlton, 1973). Another is the observation that pigment concentration in melanophores of *Uca pugilator* is inhibited by high concentrations of colchicine (Lambert and Crowe, 1973), but similar concentrations of lumicolchicine, the UV-light derivative of colchicine which does not bind tubulin, have been shown to be equally effective in inhibiting pigment concentration (Lambert and Fingerman, 1976). If colchicine were acting by disrupting microtubules, then one would not expect lumicolchicine to mimic its action.

The ionophore studies reported here and elsewhere (Lambert and Fingerman, 1977) also tend to discount microtubular involvement in pigment concentration in crustacean chromatophores. It is apparent from these studies that an influx of Ca^{++} causes pigment concentration, yet Ca^{++} has been found by Weisenberg (1972) to inhibit microtubule polymerization and by Haga, Abe and Kurokawa (1974) and Gaskin, Cantor, and Shelanski (1975) to cause disassembly of existing microtubules. In fact, the ionophore A23187 has recently been employed by Schliwa (1976) to cause shortening of the axopodia of the heliozoan, *Actinosphaerium eichhorni,* this shortening being accompanied by a Ca^{++}-mediated depolymerization of microtubules. Since colchicine inhibits pigment concentration in crustacean chromatophores, whereas ionophore-induced Ca^{++} influx causes pigment concentration, and since both agents depolymerize microtubules, it is difficult to see how microtubules can be involved in pigment concentration in these chromatophores.

As explained below, employing the ionophore in conjunction with colchicine has helped to localize the site of action of the latter. When erythrophores of *Palaemonetes pugio* were treated with colchicine before exposure to A23187, it was found that the colchicine had no effect on ionophore-induced pigment concentration (Fig. 3). It appears then that the high concentrations of the drug necessary for its inhibitory action on hormone-induced pigment concentration (Fingerman *et al.*, 1975) may in fact be interfering with the initial events of hormone action—those preceding Ca^{++}-mediated actions and not involving microtubules. One target would be the plasma membrane, since colchicine has been found to interact with non-microtubular plasma membrane proteins of other cells (Mizel and Wilson, 1972a; Wunderlich, Müller, and Speth, 1973; Bos and Emmelot, 1974; Stadler and Franke, 1974; Furcht and Scott, 1975). Exactly how the drug would interact with the chromatophore membrane remains to be determined, however. The earliest action that it might have would be interfering somehow with the hormone's interaction with its receptor. Another possibility would be events immediately following the binding, such as those mediated by the movement of integral proteins within a fluid mosaic membrane (Singer, 1974). If, as we have suggested earlier (Lambert and Fingerman, 1977), RPCH acts in conjunction with its receptor as an ionophore, high concentrations of colchicine may prevent proper association of the integral proteins which, upon binding RPCH, normally form the ionophore complexes.

It should be noted that, whereas colchicine does not inhibit A23187-induced pigment concentration, it does inhibit somewhat the dispersion of Ca^{++}-concentrated pigment that normally results from the removal of Ca^{++} from erythrophores via an EGTA/ionophore saline (Fig. 3). Hence, the possibility remains that microtubules could be involved in pigment dispersion, although the inhibition observed may have simply been due to non-specific poisoning by the long exposure to the high concentration of the drug.

While it seems clear that colchicine does not inhibit pigment concentration in crustacean chromatophores via disruption of microtubules, it is premature to extend this interpretation to the chromatophores of vertebrates: the results of the sulfhydryl experiments reported here, contrasted with those of Wikswo and Novales (1972b), indicate that colchicine may act on different targets in vertebrates and crustaceans. These investigators reported that both glutathione and cysteine hydrochloride prevent colchicine from inhibiting pigment concentration in melanophores of *Fundulus heteroclitus*. In contrast, these agents have no such effect when applied to the melanophores of *Uca,* even at concentrations much higher than those used by Wikswo and Novales. There is, however, some similarity between the melanophores of these two species: pigment concentration is inhibited by mersalyl acid in both cases, and this is prevented by glutathione (Figs. 1, 2).

CB has now been found to inhibit A23187-induced pigment concentration in prawn erythrophores (Fig. 4) in addition to its reported effects on RPCH action (Robison and Charlton, 1973; Fingerman *et al.,* 1975). It is of possible interest that the drug also inhibits ionophore action on amphibian melanophores, where the ionophore induces pigment dispersion rather than pigment concentration (Novales, 1977). In the present experiment, the effects of CB could not be reversed simply by exposing the erythrophores to fresh saline lacking the drug. A similar delay in reversibility was reported by Fingerman *et al.,* (1975) for CB inhibition of RPCH-induced pigment concentration. Only after a second medium change, to saline now containing RPCH (Fingerman *et al.,* 1975) or A23187 (Fig. 4, III), do the erythrophores recover their ability to concentrate their pigment. The delayed response may be due to the time or number of washes necessary for the cytochalasin to diffuse out of the cells to yield an intracellular concentration low enough to permit pigment movement. Alternatively, the additional stimulus by the second application of hormone or ionophore may be required to override the drug's inhibitory action. Since CB inhibits both hormone- and ionophore-induced pigment concentration, it seems probable that it is acting on the pigment concentrating machinery itself. A similar situation may apply to the pigment dispersing machinery: Lambert and Crowe (1973, 1976) have found that CB inhibits both hormone- and cAMP-induced pigment dispersion in melanophores of *Uca pugilator,* indicating that its action is exerted at some step beyond the postulated hormonal activation of adenylate cyclase, possibly at the level of the pigment-dispersing machinery. It is possible, therefore, that the same organelles may be involved in both pigment dispersion and concentration.

In contemplating the nature of these organelles, the rapid Ca^{++}-mediated contraction of muscle, as well as the possible control by Ca^{++} of actomyosin systems in many non-muscle cells (see Clarke and Spudich, 1977, for recent review) comes to mind. It is worth noting that, while CB has a variety of other actions, there is good recent evidence that the drug interferes with the actin networks of several non-muscle cells (Weihing, 1976; Hartwig and Stossel, 1976; Weber, Ranthke, Osborn and Franke, 1976; Osborn, Franke, Weber, 1977). To date, attempts to convincingly demonstrate the presence of actin filaments in crustacean chromatophores have been unsuccessful, although this may simply be due to technical problems. It is worth noting, however, that there is a precedent for the occurrence of non-actin, non-microtubule systems in cellular movement: the contractile organelle of vorticellid ciliates, the spasmoneme, consists primarily of proteins, called spasmins, which appear not to be actin (Routledge, Amos, Gupta, Hall, and Weis-Fogh, 1975; Amos, Routledge, and Yew, 1975). Interestingly, these spasmins bind Ca^{++}, and this binding apparently results in a conformational change in the proteins, culminating in contraction of the entire organelle. It is tempting to speculate that the filamentous matrix seen interconnecting the pigment granules of crustacean chromatophores might behave in an analogous manner, Ca^{++} binding to the subunits of the network, causing conformational changes which lead to contraction of the network, and hence pigment concentration. In this regard, Byers and Porter (1977) have recently described a three-dimensional "trabecular network" interconnecting the pigment granules in erythrophores of *Holocentrus ascensionis.* This network appears to contract upon pigment concentration, pulling the granules with it. A similar network has been reported by Schliwa and Euteneuer (1978) to be present in the melanophores of *Pterophyllum scalare;* these latter investigators suggest that this network may be the principal agent involved in pigment granule concentration in fish chromatophores, the microtubules perhaps assisting in guiding the pigment movement. So far as pigment dispersion in crustacean chromatophores is concerned, one might speculate further that microtubules are involved, since colchicine inhibits pigment dispersion induced by red pigment-dispersing hormone (RPDH) (Fingerman *et al.,* 1975) and, possibly, by A23187/EGTA-mediated Ca^{++} efflux.

In keeping with the scenario proposed earlier (Lambert and Fingerman, 1977), that RPCH acts via Ca^{++} influx and RPDH via cAMP production, the following heuristic model of hormonal control of pigment granule translocation is proposed. Upon RPDH binding, adenylate cyclase is stimulated and the resultant cAMP increase stimulates microtubule polymerization. The elongating microtubules push the pigment granules out into the processes of the cell, either directly or via the filamentous matrix. Upon binding the opposing hormone, RPCH, Ca^{++} enters the cells and binds to the filamentous matrix (whatever its makeup), causing it to contract and pull the pigment granules toward the center of the cell. In addition, the Ca^{++} influx depolymerizes the microtubules, further enhancing pigment concentration. RPCH action would, therefore, easily override that of RPDH. Redispersion of pigment would occur when RPCH is removed, allowing the intracellular Ca^{++} level to return to its low "resting" value and RPDH-increased cAMP levels to re-stimulate microtubular polymerization. This model, while merely a working hypothesis, contains a number of testable ideas, and may prove useful in further examination of the physiology of crustacean chromatophores.

We would like to thank Dr. R. Hamill for the generous gift of the ionophore; Dr. George Newport for valuable discussions; Dr. Francis Gress for access to the electron microscope; Justine Gillen and Darcy Fazio for assistance in preparing this manuscript; and Dr. David Burkholder for proof-reading. This investigation was supported by Grants BMS75–03029 and PCM76–82734 from the National Science Foundation.

SUMMARY

1. Using the prawn, *Palaemonetes pugio,* and the fiddler crab, *Uca pugilator,* the modes of action of cytochalasin B and colchicine in inhibiting pigment granule movement were further characterized.

2. Colchicine was unable to inhibit, in ovarian erythrophores of *Palaemonetes pugio,* pigment concentration caused by the ionophore A23187. It did, however, inhibit somewhat the pigment dispersion that normally occurs upon placing A23187-treated erythrophores in Ca^{++}-free, EGTA-containing saline.

3. Inhibition by colchicine of pigment concentration in melanophores of *Uca pugilator* was not prevented by high concentrations of glutathione or cysteine hydrochloride. Pigment concentration was inhibited by mersalyl acid, and this was prevented by glutathione.

4. Cytochalasin B inhibited ionophore A23187-induced pigment concentration in ovarian erythrophores of *Palaemonetes pugio.*

5. Electron microscopy revealed a filamentous matrix interconnecting the pigment granules of *Uca pugilator.*

6. Speculation is presented as to the modes of action of cytochalasin B and colchicine, and a heuristic model for the control of pigment movement is put forward.

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VISUAL BEHAVIOR, EYE AND RETINA OF THE PARASITIC FISH *CARAPUS MOURLANI*

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During the 1975 "Alpha Helix" South East Asia Bioluminescence Expedition to the South Moluccan Islands, three species of carapid fishes (*Carapus homei, C. parvipinnis,* and *C. mourlani:* Meyer-Rochow, 1977) were caught. All three inhabit various sea-cucumbers or large starfish of the genus *Culcita.* For many species it is still unknown how often and whether they leave their host at all for feeding, but there is now, at least for *C. mourlani,* some evidence that this species is a parasite which not only obtains shelter from its host but also devours its gonads and gut diverticula (Jangoux, 1974).

A report by Mortensen in 1923 indicated that carapids must be very abundant in the South Moluccas, but no specimens were taken during the 1973 Rumphius Expedition I (Springer, Burhanuddin and Gomon, 1974). Presumably because of their nocturnal and cryptic behavior these fishes often remain unnoticed (*e.g.,* local Banda fishermen were apparently unaware of the occurrence of fish inside starfish and often watched with great curiosity when we opened a *Culcita* during the "Alpha Helix" expedition). The rate of infestation of *Culcita* by *C. mourlani* is remarkably high: Mortensen (1923) found every second starfish infested, and Meyer-Rochow (1977) reports a slightly lower value of 1 fish per 2.1 starfish.

The behavior of these unusual fishes, which are popularly known as inquiline or intestine fish, pearlfish or assfish, has attracted the attention of natural historians (Emery, 1880; Arnold, 1953; Trott, 1970), ichthyologists (Smith, 1955; Arnold, 1956; Trott, 1970) and physiologists (Seymour and McCosker, 1974) for a long time. Greenwood, Rosen, Weitzman and Myers (1966) suggest that on the basis of morphological and ecological factors, pearlfishes form one of three families of the sub-order Ophidoidei, which are placed with the gadiform fishes. Courtenay and McKittrick (1970), after comparing sound-producing mechanisms in these fishes, come to the same conclusion and confirm the close affinities between carapids and ophidiids.

To the best of the author's knowledge the eye and retinal organization of a carapid fish have never before been studied in detail, although the adult eye (Nicol, Zyznar, Thurston and Wang, 1975) and larval eye (Meyer-Rochow, 1972) of related ophidiids have been examined. However, on the question of whether carapids use their photoreceptors at all, some information is available. Trott (1970) in *Jordanicus gracilis* and Meyer-Rochow in *C. mourlani* (this paper) have observed that these two species exhibit pupillary contraction after exposure to bright light. Pupillary reflexes among fishes are rare and seem confined to primarily crepuscular or nocturnal species (*e.g.,* eel). Although a carapid fish seems to employ olfaction and touch in locating the entrance to its host, be it anus (in case of holothurian) or mouth (in case of the starfish), vision, too, plays a part.

It can easily be demonstrated that a fish which is isolated from its host prefers a dark place and swims to the darkest corner of the aquarium. The fish also follows the starfish when the latter is moved about behind a glass division. *C. mourlani* also swims into a hollow human fist, when a little entrance large enough to allow the fish's passage, is left. These observations, which were carried out on freshly caught animals in aquariums on Groot Banda, clearly indicate that vision, in spite of the thick outer skin which covers the eyes of *C. mourlani,* cannot be totally useless to this species. In order to find out how the animal uses its photoreceptors, the eye and structural organization of the retina had to be investigated.

Materials and Methods

During the 1975 "Alpha Helix" South East Asia Bioluminescence Expedition the eyes of five *Carapus mourlani* were prepared for this study. The fish were recovered from the coelomic cavity of large cushion starfish (*Culcita novaeguinaea*), which were collected by free-diving in shallow water between Goenung Api and Groot Banda (Banda Islands, Indonesia).

The eyes were prefixed for 12 hr in 3% sea water/0.2 M cacodylate-buffered glutaraldehyde, washed several times in a mixture of 50 ml filtered sea water, 20 ml Na Cl, 10 ml 0.2 M Na-cacodylate and 0.05 ml $CaCl_2$, and postfixed for 2 hr in 1% OsO_4. The specimens were then dehydrated in a graded series of acetone, embedded in Spurr's epoxy resin and hardened for 2 days at 65° C.

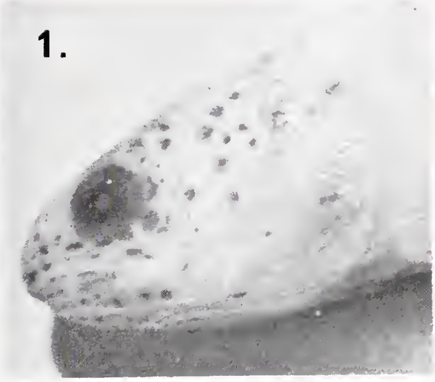
Reichert ultramicrotomes were used to cut sections for both light microscopy (stained with toluidine-blue for approximately 1 min) and transmission electron microscopy. The ultrathin sections were double-stained with uranyl acetate (20 min) and lead citrate (3 min) and observed with either a Philips 200 or a Philips 300 electron microscope.

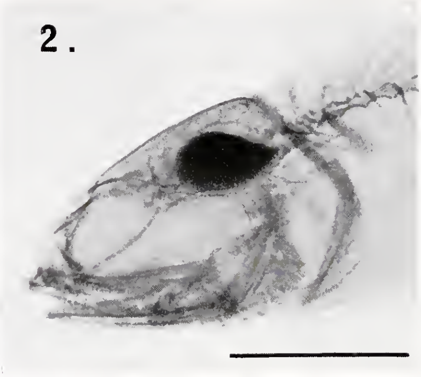
Results

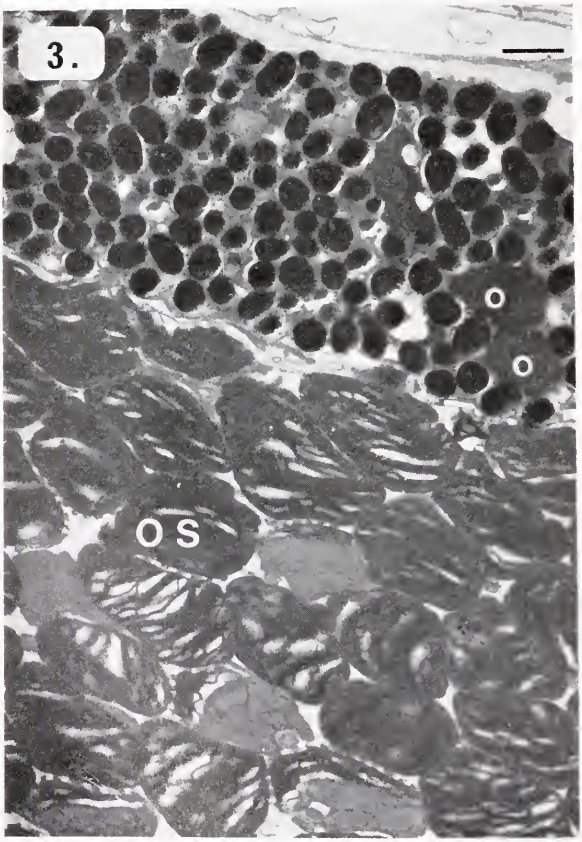
General morphology

In a 160 mm (total length) specimen of *C. mourlani* the eye is slightly oval in shape with an anterior/posterior long axis of 4.2 mm and a dorso ventral shorter axis of 3.6 mm (Fig. 1). The eye appears to grow in proportion to increased total body length, but epidermal cell layers, due to their increasing thickness and opacity, must impair vision more severely in aged specimens. The pupil in a light-adapted specimen measures approximately 0.5 mm in diameter, but can dilate to at least 2.5 times this value in complete darkness. The iris in a fresh, living specimen is of an attractive gold-metallic color. Eyeshine in dark-adapted eyes was not observed. If it occurs at all, it must be very weak and of short duration.

As in other fishes, the orbit, which in a 160 mm specimen measures approximately 5 mm in diameter, is surrounded by orbito/temporal skull bones (Fig. 2), of which alisphenoid and orbitosphenoid form the inner wall. On the outside the orbit is surrounded by supraorbitale, dermosphenoticum and other dermal infraorbitalia, all of which are of surprisingly firm consistency. This may be interpreted as an adaptation to the pressures that this region of the head has to withstand







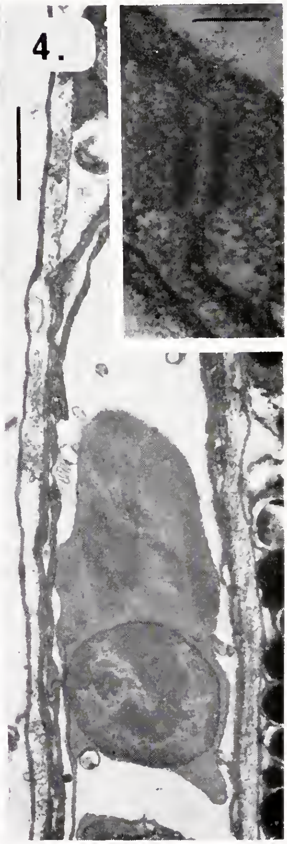


FIGURE 1. Close-up photograph of the head of a 160 mm (total length) *Carapus mourlani*. Apart from a few scattered melanophores, the fish is pale. The eyes lie under a relatively thick layer of cream-colored, semi-transparent skin.

FIGURE 2. Otoliths, skull bones as well as the orbital space show up clearly in this X-ray photograph. The scale, which is also applicable to Figure 1, is 1 cm.

when the animal penetrates anal or oral openings of holothurian and starfish, respectively.

The lens is spherical and has a diameter of 0.7 mm in a 80 mm individual. It consists of long, densely-packed living cells. The distance between retina and lens center is, on average, two times the lens radius. The choroid rete mirabile is inconspicuous and presumably reduced, as in deep-sea forms (for more detailed information on the question of blood supply to the eye of fishes see publications by Wittenberg and Haedrich, 1974, and Copeland and Brown, 1976).

The retina and associated structures

Capillaries. The retina is well-supplied with capillaries. Capillaries of varying diameter can be see within the retina and on the inner surface of the retina as well. Blood cells and their nuclei are often discernable in sections of retinal capillaries (Fig. 4). Fish eyes do not have, in general, the massive network of capillaries seen in the mammalian eye. However, from the work of Ali, Anctil and Mohideen (1968) we know that capillaries are not uncommon on the inner surface. Capillary junctions, rather than being developed as "zonulae occludens" as in most mammals, resemble desmosomes of 0.14 μ length (Fig. 4). This may possibly be interpreted as a primitive form of capillary junction and would be worth following up further.

Sclera and cornea. The cornea consists of two major layers, which are closely connected. They are the dermal cornea, which is the modified skin covering the eye ball, and the scleral cornea, which is continuous with the cartilaginous sclera and which consists of an inner and an outer component. The dermal skin exhibits progressive corneal keratosis with increasing age and must impair vision in older specimens because of its thickness and milkiness.

Choroid and pigment epithelium. Choroid and pigment epithelium, which do not possess any obvious processes in *C. mourlani*, are conventional in their gross anatomy. Pigment cells and their uniformly-stained nuclei are relatively large. Large numbers of dark inclusions of varying sizes are present in the epithelial cells (Fig. 3). The size/frequency distribution of these objects suggests that three groups may be distinguished: tiny, globules of an average diameter of 0.17 μ; large, more or less spherical bodies of approximately 0.5 μ diameter; and ovocylindrical to cylindrical structures, about 0.5 μ in diameter and up to two microns in length. However it must be pointed out that size, abundance and distribution of granules are often linked to functional state or age of the cell, and any categorization of granules based on these parameters alone must be tentative (Fineran and Nicol, 1974). In spite of their reddish brown color in fresh specimens, all types of melanosomes are extremely electron opaque. Tapetal spheres,

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FIGURE 3. The pigment cell layer is planar and screening pigment granules do not penetrate between outer segments (OS). Less densely-strained structures (white circles) may represent lipid bodies. The scale line is 1 μ.

FIGURE 4. A capillary within the retina with blood cells and nuclei visible. The scale line is 1 μ. The inset shows an unusual desmosome-like junction between two retinal capillaries, which may possibly be interpreted as a primitive form of capillary junction. The scale line is 0.1 μ.

which are often difficult to distinguish from spherical melanosomes, have been reported from ophidiid eyes and may also occur in *C. mourlani.* Guanine crystals, however, seem to be absent and holes in the retina, rather than being indicative of guanine material (Arnott, Best, and Nicol, 1970), are more likely to represent a reflection of the difficult fixation properties of the *C. mourlani* retina.

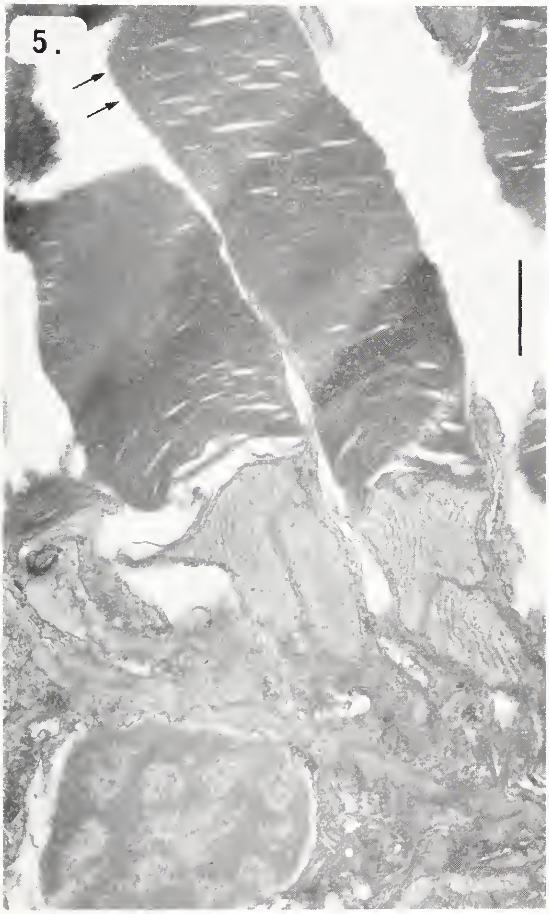
Even though lipid tapeta have been found in the eyes of ophidiids (Arnott, Maciolek and Nicol, 1970), it is doubtful whether less darkly stained circular to ovoid structures with diameters of between 1 and 2 μ represent lipid bodies, for they are not very abundant (Fig. 3) and their density is no greater in the very short inward-extending processes (Nicol *et al.,* 1975). Mitochondria, exhibiting circular or elongate (0.8 × 1.5 μ) profiles (indicating a cylindrical shape), were present, as well as what Fineran and Nicol (1974) term occasional "myeloids" (here: stacked membranes and smooth endoplasmic reticulum). The pigment epithelium in *C. mourlani* is planar (as in deep-sea fishes), and the pigment is likely to be stationary and confined to the cell bodies of the pigment epithelium as in other ophidiids.

Outer segments. The outer segments of *Carapus* photoreceptor cells seem anatomically to be of one type only. Twin or double receptors as in labrid species (Fineran and Nicol, 1974) or *Scopelosaurus lepidus* (Munk, 1977), or grouped units, commonly found in the eyes of deep-sea fishes (Locket, 1970, 1971), do not occur, but a certain degree of banking with up to four tiers is observable.

The outer segment layer is 20 μ wide, and each outer segment is approximately 5 μ long and 1 μ thick (Fig. 5). The density per 10 × 10 μ is 40, which corresponds to a value of 400,000 mm. Although a treatment of the retina with ZIO to distinguish unambiguously between rods and cones (Pellegrino de Iraldi, 1972) was impractical at the time, the outer segments were identified as rods using criteria recently summarized in a paper by Munk (1977). Individual rods are neither isolated from each other by envelopes of screening pigment nor are they surrounded by concentric layers of reflecting crystals (Figs. 3, 5, 6). Close contact between membranes of two or three different outer segments is, therefore, possible. Approximately 15 calycal processes, which are inner segment projections (Pietzsch-Rohrschneider, 1976), run alongside each outer segment, but do not interfere with intersegmental contact (Fig. 6). A lateral sac (Fineran and Nicol, 1974) or "accessory outer segment" (Yacob, Wise and Kunz, 1977) is not developed and obvious outer segment incisures (as in *Anableps anableps:* Borwein and Hollenberg, 1973) have not been observed with certainty. The cylindrical outer segment is made up of stacked lamellae, the spacing of which was found to be 15 nm (Fig. 5). The membranes of the lamellae are not continuous with cell membranes; they are closed and form unusually thin discs of 10 nm thickness (Fig. 5).

Inner segments. The inner segment layer is approximately 10 cells thick. Outer and inner segment, which are in direct opposition, are connected by a short cilium (Fig. 6). This shows the 9 + 0 filament arrangement typical of other visual cell cilia (Locket, 1971) and arises presumably from a centriolar body. The cilium is eccentrically placed and surrounded by a clear space, but a regular pattern in which the position of the cilium is fixed is not apparent. A second centriol was not found.

The ellipsoid is fairly short (approx. 6 μm) and possesses a cross-sectional diam-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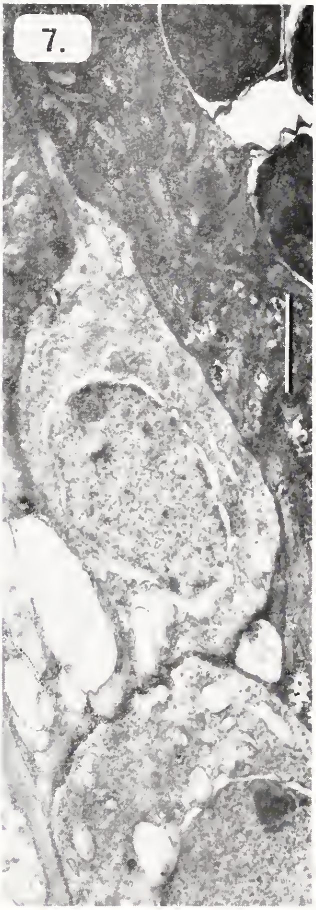
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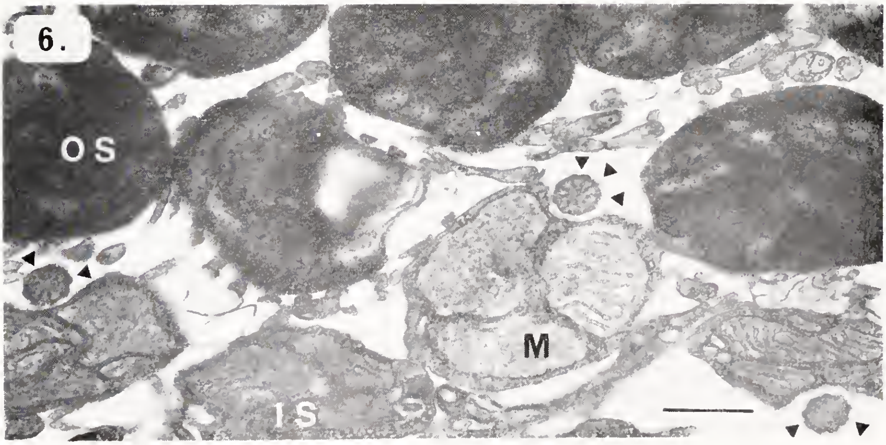
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eter of the same order as that of the outer segment (Fig. 5). The mitochondria are largest (up to 1 μ in diameter) and most numerous at the distal end. The most sclerad-positioned mitochondria often exhibit an unusual multilamellated internal fine-structure (Schoebitz, Rodriguez-Echandia and Campos, 1973). Further vitread the mitochondria are separated from each other by fine granular cytoplasm.

The nuclei appear as mottled cylindrical organelles with rounded ends below the mitochondria. In this plane the membranes of adjacent receptor cell bodies often touch each other and may give rise to tight junctions, but whether true synapses occur is unknown.

Outer plexiform. The outer plexiform layer represents a uniform network of processes of the same dimeter (0.5 μ). These originate from a variety of cells including Muller and horizontal cells. No typical spheroids or conoids were seen, but as the fixation of this layer was poor, few valid conclusions can be drawn. Synapses do occur, but they seem atypical.

Biopolar cells and inner nuclear layer. The inner nuclear layer consists of only 1 or 2 layers of nuclei, which belong to the bipolar, amacrine, and horizontal cells. Bipolar cells are characterized by relatively large, mottled nuclei with reticulated chromatin patterns. Their plasma contains mitochondria and other normally occurring subcellular components such as ribosomes, endoplasmic reticulum, vesicles, and granules which may be glycogen (Locket, 1971).

Inner plexiform layer, ganglion cell layer and outer nerve fiber layer. The inner plexiform layer is unusually wide, and, although badly fixed, allows identification of some amacrine synapses. There are very few ganglion cells (Fig. 7) and the ratio of nuclei in the outer nuclear layer to those of the inner nuclear layer to ganglion cells is approximately 100:10:1. A similar ratio has been reported for the deep-sea fish *Coryphaenoides rupestris* by Penzlin and Friedrichsen (1973). Very few optic nerve fibers are present, but there are definitely some. The output from the retina must be small and resolution is expected to be poor.

Lens muscle. The eye of *Carapus mourlani* possesses a typical ventrally-located ectodermal lens muscle. The lens muscle, which originates from the inside of the iris close to the *ora terminalis retinae* (Munk, 1971), consists of smooth fibers.

DISCUSSION

The eye of *Carapus mourlani* is an interesting sense organ, because it combines features of degenerated photoreceptors characteristic of cave organisms (keratosis, small number of nerve fibers, no screening pigment between the rods) with adaptations which are commonly met with in predominantly nocturnal and

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FIGURE 5. Slightly oblique radial section through outer segment and ellipsoid portion of photoreceptor cell. The photoreceptor cells are banked, but they are of one type only. The membranes of the lamellae are not continuous with cell membranes (arrows); they are closed and form unusually thin discs. The scale line is 1 μ.

FIGURE 6. Transverse section through outer/inner segment junction with asymmetrically-placed connecting cilia clearly visible (arrow heads). Approximately 15 calycal processes which are inner segment (IS) projections, run alongside each outer segment (OS).

FIGURE 7. Section through two ganglion cells and their nuclei. The inner plexiform layer is unusually wide in the eye of *C. mourlani*, but the number of ganglion cells is very small, indicating a high degree of convergence. The scale line is 1 μ.

deep-sea forms (*e.g.,* pure rod retina, pupillary contraction, receptor-bipolar-ganglion cell ratio).

Presumably, the eye is fully functional in the larval and juvenile stages of *C. mourlani,* but degenerates with increasing age. Age-dependent retinal degenerations have been reported, for example, in the photoreceptors of the Ozark cave salamanders (Besharse and Hollyfield, 1977), and in the eye of the cave fish *Anopthichthys* (Kuhn and Kähling, 1954). The retinal structure of *Carapus* suggests that the eye of the latter is adapted to a dark environment, and that, although it must have poor resolution, it has become modified as a receptor for increased sensitivity in low environmental brightness conditions. It is believed that the visual performance of larval *Carapus* fish is better than that of adults, which spend a considerable amount of time inside holothurians, where light levels are so low that they could not be detected by sensitive light-meters (Trott, 1970).

The pupillary change, on the other hand, which can be observed in fresh specimens removed from their hosts, is direct evidence that these fish are still sensitive to different light levels. Unfortunately, it is not known at this stage whether the retinal pigment in *Carapus* migrates, at it does in many other freshwater and marine fishes (Nicol, 1965; Lang, 1965; Roeser, 1973).

Another unsolved problem concerns the formation of an image on the retina. It is generally accepted that the fish eye lens forms a good image across the full aperture due to a radial gradient of refractive index, with higher values in the center and lower ones near the periphery (for theoretical mathematical treatment see Fletcher, Murphy and Young, 1954). However, it is still an unsettled question as to whether fish eyes exhibit myopia or emmetropia (Munk, 1971; Sivak, 1976). Penzlin and Rönicke (1976) point out that, in spite of the common use of the terms "myopia", "emmetropia", and "hyperopia", these are misleading when applied to fish eyes, for one and the same eye may be both myopic and emmetropic, depending on the direction the light strikes the lens.

The lens, as in almost all fishes, is spherical in *Carapus* and cannot change its curvature. The quotient of retina-lens distance and lens radius is approximately 2 and, thus, comes close to the often cited "Mathiesen ratio" of 2.55. Ray paths and image formation, however, will have to remain speculative until more data are available on lens refractive indices.

At this stage one may, therefore, conclude that the eye of *C. mourlani* is basically a photoreceptor designed for vision in a dark environment characterized by high sensitivity and poor resolution, but that its visual performance drops with age. The age-dependent deterioration of vision is likely to be connected with the "pseudo-cave" conditions which must prevail inside the dark coelom of the fish's echinoderm host.

The authors wish to thank Dr. Leo Sosula (Prince of Wales Hospital, Randwick, N S W, Australia) for his aid in this research, and the Director of the Meat Industry Research Institute for use of the Philips EM200. Thanks are extended to Dr. Jim Chalcroft and Mr. Gary Leet for their efficient maintenance of the instrument, and the staff of the Waikato Hospital Mammography Unit for the preparation of the X-ray photograph, Figure 2 in this paper.

SUMMARY

1. This is the first transmission electron microscope description of the retina of a parasitic fish.

2. The retina is well supplied with capillaries, some of which occur on the inner surface of the retina. An unusual and possibly primitive type of capillary junction is described.

3. The outer segments of the retina of *Carapus mourlani,* which measure approximately 2 μ in diameter, are of one type only. A certain degree of banking was observed. Outer segments are not isolated from each other by screening pigments; they are, however, surrounded by about 15 calycal processes.

4. Typical spheroids or conoids were not seen in the outer plexiform layer. The inner nuclear layer consists of only one or two layers of nuclei. The inner plexiform layer is unusually wide and some amacrine synapses can be identified. There are few ganglion cells and the ratio of nuclei in the outer nuclear layer to those of the inner nuclear layer to ganglion cells is approximately 100:10:1.

5. The eye of *C. mourlani* combines features of degenerated photoreceptors, characteristic of cave-organisms, with adaptations which are commonly found in nocturnal and deep-sea forms.

6. The visual behavior of the animal indicates that the eye is fully functional in spite of its corneal keratosis and small number of optic nerve fibers.

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AN EXPERIMENTAL GRADIENT ANALYSIS: HYPOSALINITY AS AN "UPSTRESS" DISTRIBUTIONAL DETERMINANT FOR CARIBBEAN PORTUNID CRABS

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Gradients provide opportunities to examine the responses of populations to their environments, and their contributions to community structure. Interpreting observations from gradients is easiest when a single important physicochemical factor varies monotonically. When no major physicochemical variable is monotonic, two or more actual gradients result. For example, in North America, peak air temperatures decline both north and south from mid-continent maxima (MacArthur, 1972). Alternatively, when more than one major physicochemical variable is monotonic, as in the altitudinal gradients studied by Terborgh (1971) and Whittaker (1967), their effects cannot be unraveled by observation alone. Since many, perhaps all gradients are complex (those Whittaker, 1967, defined as having multiple physicochemical variables), experimental manipulations permit isolation of variables, greatly increasing the utility of gradient analyses.

This study examines some determinants of swimming crab distributions along gradients in terrestrial influence on aquatic climate having monotonically varying salinities. Most of the ~ 300 species in the family Portunidae inhabit shallow tropical marine waters (Rathbun, 1930; Stephenson, 1972), but crabs in the Indo-West Pacific genus *Scylla,* and the Atlantic and East Pacific genus *Callinectes,* inhabit climatically more rigorous biotopes, such as rivers and estuarine bays. The weather of waters in most portunids' habitats is less influenced by aerial weather over the nearest landmasses than is aquatic weather in the habitats of *Scylla* and *Callinectes.* We will focus on a functionally similar grouping, or guild (Root, 1967) of Caribbean species in the largest portunid subfamily, the Portuninae. The guild members are demersal, in and epifaunal carnivores or carnivore-detritivores with long-lived planktotrophic larvae. They had been studied mainly taxonomically, with the exception of *Callinectes sapidus* Rathbun, a commercial species which controls community structure of estuarine benthos in Chesapeake Bay, USA (Virnstein, 1977). *C. sapidus* occurs from fresh through marine and even hypersaline waters (Mangum and Amende, 1972; Simmons, 1957). It is catadromous; females migrate from hyposaline waters (having less than about 35‰ salinity) to higher salinities to spawn and hatch their eggs (Churchill, 1919; Van Engel, 1958). The planktonic early zoeal larvae are orthostenohaline (Kalber, 1970), while subsequent megalopae and crab stages, which reinvade estuaries, are progressively more euryhaline (Costlow, 1967; Tagatz, 1971).

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Scattered previous records (Coelho, 1965, 1967; Holthuis, 1959; Rouse, 1969; Taissoun, 1973) and inferences from geographic distributions (Norse, 1977) indicated that salinity is a major niche axis along which Caribbean portunid species are differentially distributed. For this originally marine group, rivers (in which salinities are constantly stressful) and nearshore shallows (where salinity stress is highly variable and unpredictable) should mark "upstress" ends of species' distributions, which extend through more equable waters to seaward. "Upstress" is the direction of increasing physiological stress directly attributable to physicochemical change. Differential upstress limits for Caribbean portunids should therefore reflect varying tolerances of hyposalinities. To test this, experiments in this study simulate acutely diluted conditions portunids can encounter in the study areas. Norse (1978) and Norse and Estevez (1977) considered some factors limiting portunids' "downstress" distributions.

Jamaica was the main study area for field and laboratory work. The number and extent of Jamaican hyposaline biotopes depend more on upland, than local rainfall; *e.g.,* the Martha Brae, one of the largest rivers, feeds a large brackish bay in one of the island's driest regions. Most work was done on the North Coast, where the shelf is narrow, and shallow soft bottoms suitable for most Caribbean portunids occur in relatively small pockets in which waters may grade from riverine to oceanic in a few hundred meters.

Supplemental field work was done in the Middle Florida Keys, USA, in 1972 and 1974, in Colombia between the Ciénaga Grande de Santa Marta and Bahía Concha in 1972, and in Curaçao in 1975. Descriptions of sites in Jamaica, Colombia and the Florida Keys appear in Norse (1975).

Materials and Methods

Distributions

Crabs were sampled in coastal and lotic waters by trapping, hand-netting and gill-netting. To minimize bias, crabs were hand-netted in the order in which they were spotted.

Thirty-two Jamaican quantitative sites were sampled from 1 to 13 times in July to August, 1970 and May to October, 1972. Crabs were sorted to species and sex and measured (carapace length, CL, along midline) immediately after capture. Water samples were taken just above the substratum in the middle of collecting sites. Most salinities were measured with an American Optical salinity model refractometer to the nearest $\frac{1}{2}$‰; a few were determined by titration. Supplemental collections included those in which crabs from adjacent habitats were pooled, less than five crabs were taken, or particular species, sexes or sizes were sought for experiments.

Hyposalinity tolerances

Jamaican *Callinectes maracaiboensis* Taissoun, *C. bocourti* A. Milne Edwards, *C. sapidus, C. exasperatus* (Gerstaecker), *C. danae* Smith, *C. marginatus* (A. Milne Edwards), *C. ornatus* Ordway, *Arenaeus cribrarius* (Lamarck) and *Portunus sebae* (H. Milne Edwards) were brought to Discovery Bay Marine

Laboratory in 1972. Although aware that Taissoun (1972) was describing a new species (*C. maracaiboensis*), I was unaware that its range included Jamaica (see Norse, 1977), and unfortunately, did not distinguish it from the morphologically very similar *C. bocourti* in any laboratory experiments. For convenience, they will be referred to as "*C. bocourti*" where appropriate. Crabs collected below 20‰ were placed in stepwise increasing salinities for several hours. All were acclimated in running seawater (salinity unvaryingly 34.5‰; mean temperature 28.9° C) for a minimum of 30 hr. Healthy hard-shelled crabs were placed singly into covered, individually aerated plastic buckets containing 1 to 3 liters (according to crab size) of water prepared by diluting 100% seawater with unchlorinated tapwater to 50, 25, and 10% seawater concentrations. Salinities were measured and adjusted where necessary. Three hundred eighty crabs were tested, and after 24 hours, examined for survival. "Living" crabs moved appendages and responded to prodding, although some were obviously stressed and probably would not have survived continued dilution.

Further experiments were done in the Florida Keys in July, 1974 at Pigeon Key Marine Environmental Station. *Callinectes ornatus*, *Portunus depressifrons* (Stimpson), *P. spinimanus* Latreille, *P. gibbesii* (Stimpson) and *Cronius tumidulus* (Stimpson) were collected near Ohio (= Sunshine) and Missouri Keys. Acclimation salinities averaged 36.3‰ and varied no more than 1‰ from this value; temperatures averaged 27.9° C. This time, crabs were placed in 1 liter of seawater diluted with aged tapwater to 50, 37.5, and 25% seawater concentrations; 264 were tested. Experiments were otherwise similar to those in Jamaica.

RESULTS

Distributions

A total of 943 *Callinectes* belonging to eight species, and 47 other demersal portunids (five species) were collected in Jamaican quantitative samples; hundreds more belonging to 16 species were observed or collected at supplemental sites in Jamaica, Florida, Colombia and Curaçao. Two previously unrecorded species were found in Jamaica; *C. maracaiboensis* is common there (Norse, 1977), while *C. similis* Williams (3 individuals) was taken only once.

All guild members occur in marine salinities, although progressively fewer species were found as salinities decreased. Table I gives the highest and lowest salinity records (ecological amplitude—Terborgh, 1971), and the highest and lowest mean salinities of quantitative sites where each Jamaican species comprised ≧ 25% of the portunids taken, an arbitrary measure of ecological importance. *Callinectes maracaiboensis, C. bocourti, C. sapidus* and *C. exasperatus* have ecological amplitudes exceeding 33‰, while *C. danae, C. marginatus, C. ornatus, Arenaeus cribrarius* and the others have progressively smaller ones, although *A. cribrarius* and the others were found at two or fewer Jamaican sites. *C. sapidus* is ecologically important over a range of 23.9‰; diminishing ranges are seen for *C. maracaiboensis* (21.3‰), *C. bocourti* and *C. exasperatus* (19.8‰), *C. danae* (14.3‰), *C. marginatus* (8.5‰), *C. ornatus* (3.4‰) and *P. sebae* (0.0‰).

Table I also gives the means of indivdual site species compositions (dominance) for *Callinectes* in four salinity categories: "freshwater" (actually including

TABLE I

Salinity distributions of Jamaican portunids (*C. represents* Callinectes). *Ranges of occurrence and of ecological importance (in ‰).*

	Lowest	Lowest $\bar{X}$ where sp. $\geq 25\%$	Highest $\bar{X}$ where sp. $\geq 25\%$	Highest
C. maracaiboensis	0.0	0.5	21.8	35.0
C. bocourti	0.0	2.0	21.8	34.5
C. sapidus	0.0	0.5	24.4	34.5
C. exasperatus	2.0	15.5	35.3	35.5
C. danae	11.0	17.8	32.1	34.6
C. marginatus	15.0	26.8	35.5	35.5
C. ornatus	17.8	32.1	35.5	35.5
C. similis	34.5			34.5
Portunus sebae	34.5	34.5	34.5	35.3
P. anceps	34.5			35.5
P. depressifrons	34.5			34.5
Arenaeus cribrarius	30.5			34.5
Cronius tumidulus	34.5			34.5

Callinectes spp: dominance (relative abundances of species (in per cent) in 4 salinity categories; Σ each category = 100%).

	0–10.0‰	10.1–30.0‰	30.1–33.0‰	33.1–35.5‰
C. maracaiboensis	41.0	10.0	4.0	0.4
C. bocourti	34.5	10.4	0.4	0.7
C. sapidus	22.5	15.2	4.6	1.5
C. exasperatus	2.0	29.6	22.8	27.5
C. danae	0.0	16.0	23.1	1.9
C. marginatus	0.0	16.6	30.9	29.5
C. ornatus	0.0	2.4	14.2	38.0
C. similis	0.0	0.0	0.0	0.2

the lower brackish range, 0–10.0‰), "brackish" (10.1–30.0‰), "near-marine" (30.1–33.0‰) and "marine" (33.1–35.5‰). Freshwater sites are dominated by *C. maracaiboensis, C. bocourti* and *C. sapidus,* brackish sites by *C. exasperatus,* near-marine sites by *C. marginatus, C. danae* and *C. exasperatus,* and marine sites by *C. ornatus, C. marginatus* and *C. exasperatus.*

In Jamaica, males comprised 12/16 (75%) of the *C. sapidus* adults in salinities below 15‰, but only 6/29 (21%) of the adults in higher salinities. This difference is highly significant ($G_{adj} = 10.7$; $P < 0.005$). Analogous patterns were seen in the other Jamaican freshwater species, *C. maracaiboensis* (16/20 *vs.* 2/21; $G_{adj} = 19.6$; $P << 0.001$) and *C. bocourti* (13/16 *vs* 1/11; $G_{adj} = 11.8$; $P < 0.001$). Among the three freshwater species, none of the 11 adult females taken below 15‰ was ovigerous, while 7/52 (13%) in higher salinities bore egg sponges. None of the 19 ovigerous females in other Jamaican *Callinectes* occurred below 15‰. Like North Temperate *C. sapidus* populations, Jamaican *Callinectes* appear unable to reproduce in low salinities, and the freshwater species are catadromous, storing energy in low salinity ecosystems but spawning and hatching their eggs in higher salinities.

TABLE II

Hyposalinity tolerances of Jamaican portunids.

	# tested	% surviving
Transfers from Discovery Bay seawater (34.5‰) to 10% seawater		
"*Callinectes bocourti*"	24	100
C. sapidus	20	100
C. exasperatus	33	88
C. danae	36	50
C. marginatus	29	7
C. ornatus	36	3
Transfers to 25% seawater*		
C. marginatus	30	67
C. ornatus	45	22
Arenaeus cribrarius	6	0
Portunus sebae	6	0
Transfers to 50% seawater*		
C. ornatus	21	100
A. cribrarius	6	83
P. sebae	6	17

* "*C. bocourti*," *C. sapidus*, *C. exasperatus*, and *C. danae* (25% seawater) and *C. marginatus* (50% seawater) had 94 to 100% survival.

Hyposalinity tolerances

Table II shows survival of Jamaican portunids transferred from seawater to diluted seawater. There are marked differences among *Callinectes* transferred to 10% seawater (G-test for heterogeneity, $G_{H} = 148.2$ with 5 df; $P << 0.001$). Using the conservative STP *a posteriori* test procedure (Sokal and Rohlf, 1969), "*C. bocourti*" (including *C. maracaiboensis*) and *C. sapidus* are not significantly more tolerant than *C. exasperatus*, but *C. exasperatus* is significantly more tolerant than *C. danae* ($G_{H} = 12.2$; $P < 0.05$), *C. danae* is significantly more tolerant than *C. marginatus* ($G_{H} = 15.8$; $P < 0.01$), and differences between *C. marginatus* and *C. ornatus* are not significant. Transfers to 25% seawater show marked differences among *C. marginatus*, *C. ornatus*, *Arenaeus cribrarius* and *Portunus sebae* ($G_{H} = 26.2$ with 3 df; $P << 0.001$). *C. marginatus* is significantly more tolerant than *C. ornatus* ($G_{H} = 15.1$; $P < 0.005$), but *C. ornatus* is not significantly more so than the others. Transfers to 50% seawater also show marked species' differences ($G_{H} = 20.5$ with 2 df; $P << 0.001$). Two groupings, within which differences are not significant at $P = 0.05$, are: *C. ornatus* with *A. cribrarius* and *A. cribrarius* with *P. sebae*.

The lowest salinities in which the common Jamaican *Callinectes* occur, or are ecologically important (at least 25% of the congeners at the site) are very highly correlated ($P < .001$ for both) with their hyposalinity tolerances (Fig. 1).

Table III shows survival of Florida Keys crabs subjected to diluted seawater. There are marked differences among the species ($G_{H} = 70.2$ with 4 df; $P << 0.001$) in transfers to 25% seawater, with *C. ornatus* more euryhaline than the others. Neither sexes nor sizes of *C. ornatus* differ significantly, nor do *Portunus*

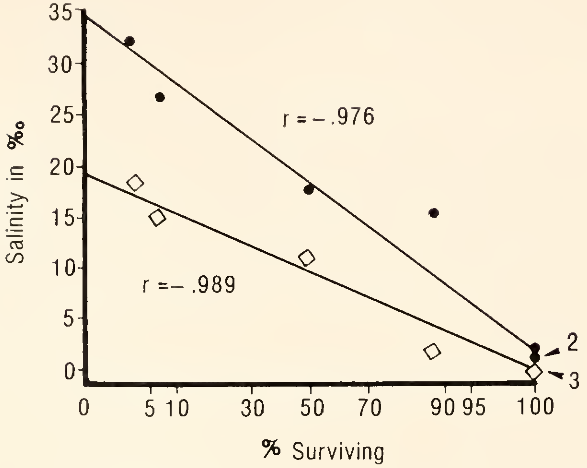

FIGURE 1. Relationship of hyposalinity tolerance (survival when transferred from seawater to 10% seawater) to lowest salinity (diamonds) and lowest salinity of ecological importance (circles) for seven common Jamaican *Callinectes* spp.

depressifrons and *P. spinimanus* when transferred to 37.5% seawater. When transferred to 25% seawater, Florida Keys *C. ornatus* are significantly more tolerant than Jamaican conspecifics ($G_{adj} = 18.0$; $P << 0.001$). In transfers to 50%

TABLE III

Hyposalinity tolerances of Florida Keys portunids.

	# tested	% surviving
Transfers from Pigeon Key seawater (36.3‰) to 25% seawater		
Callinectes ornatus	87	62
Males	53	62
Females	32	63
Sacculinized crabs	2	50
Larger crabs (>24 mm)	42	71
Smaller crabs (≤24 mm)	45	53
Portunus depressifrons	23	0
P. spinimanus	23	0
P. gibbesii	2	0
Cronius tumidulus	4	0
Transfers to 37.5% seawater		
Callinectes ornatus	18	100
P. depressifrons	30	30
P. spinimanus	21	14
Transfers to 50% seawater*		
P. depressifrons	9	100
P. spinimanus	15	100

* *C. ornatus* (50% seawater) had 100% survival.

seawater. Keys *P. depressifrons* and *P. spinimanus* are significantly more euryhaline than Jamaican *P. sebae* ($G_{II} = 27.0$ with 2 df; $P << 0.001$).

DISCUSSION

Caribbean portunids show serial replacement along gradients in terrestrial influence on aquatic climate. All 16 portunid species that were collected occur in undiluted seawater, but *Callinectes* virtually monopolize hyposaline biotopes. None of the ~290 *Portunus* or *Cronius* was found in hyposaline water, and *Arenaeus cribrarius* was taken at salinities no lower than 87% seawater. In contrast, all common Jamaican *Callinectes* occur down to 50% seawater or less. These generic distributional differences reflect differing hyposalinity tolerances. Pearse (1929) found that two *Portunus* spp. are less dilution-tolerant than *C. marginatus,* and none of the six *Portunus, Cronius* and *Arenaeus* species tested in the present study appears to tolerate acute seawater dilution as well as the least euryhaline *Callinectes*. Similar portunid distribution sequences occur along salinity gradients in the East Pacific off Colombia (Norse and Estevez, 1977). Euryhalinity permits American *Callinectes* to exploit resources in physicochemically harsh, but highly productive ecosystems from which other, potentially competing portunids (and other marine taxa) are absent.

The four to five levels of euryhalinity evolved by Caribbean *Callinectes* are strikingly correlated with species' penetration into hyposaline waters (Fig. 1). This strongly suggests a causal relationship, but does not distinguish the nature of the underlying mechanism, as Wolcott (1973) noted in his study on limpets. Upstress distributions may be directly extrinsically limited (through stress-induced death) or indirectly extrinsically limited (through death from stress-related failure to find, handle or assimilate food, escape damage in agonistic contests, evade predators or cope with parasites). Or, they may be intrinsically limited, through avoidance of low salinities.

Upstress limits may be determined by either usual or unusual weather. Available evidence indicates the latter. The extent and permanence of hyposaline biotopes differs among the study areas. Jamaica and the Ciénaga Grande de Santa Marta region in Colombia have numerous large hyposaline biotopes, while the Florida Keys, Curaçao and the area near the Colombian city of Santa Marta do not. Since rainfall in all these localities can be very intense, biotopes usually having marine salinities can undergo rapid, severe dilution. In general, likelihood and severity of dilution increase with increasing enclosure by land and decreasing depth and distance from shore. Thus, although the study areas are differentially endowed with permanent hyposaline biotopes, rapid salinity decreases can follow rains at sites in all.

Although *Portunus* were taken only in normally marine biotopes, environmental likelihood of severe dilution and euryhalinity are correlated among the species. In the Keys and Jamaica, *P. sebae* is the dominant portunid among lagoonal patch reefs and deeper, larger reefs in depths of 2 to 15 m (once at 76 cm). *P. depressifrons* in the same areas, and *P. spinimanus* in the Keys and Colombia, are common in the shallows (0–30 cm), often with *Callinectes marginatus* and *C. ornatus*. Similarly, although *P. sebae* occurs in Biscayne Bay, Florida, Park

(1969) found none in his shallow samples, which included *P. spinimanus* and hundreds of *P. depressifrons* and *C. ornatus*. *P. sebae* is less dilution-tolerant than these *Portunus* and *Callinectes*, and would seldom or never encounter substantial dilution in lagoon and fore reef ecosystems, while the others often would be subjected to mild, and occasionally severe dilution.

Another way to assess the effects of usual and unusual weather on distributions is to examine the physiology of whole assemblages. A measure of tolerance for each species can be multiplied by the species' ecological importance, and the products for all species added, yielding a weighted physiological index which can be plotted against a physicochemical variable. Unlike Whittaker's (1956), the method given below is not circular in assigning tolerances to species. A euryhalinity index (EI) for *Callinectes* (excluding *C. similis*, which is rare in Jamaica, and whose tolerances were not tested) at each site,

$$100 \sum_{i=1}^{7} \mathrm{AB}$$

where A is the proportion of species i surviving transfer from seawater to 10% seawater and B is relative abundance (dominance) of species i, is plotted against mean salinity at 31 Jamaican sites in Figure 2. Clearly, low salinity assemblages are dominated by highly euryhaline species while less euryhaline species increasingly dominate as salinity increases, but there is also a mean-variance correlation, necessitating transformation of EIs before measurement of correlation is valid.

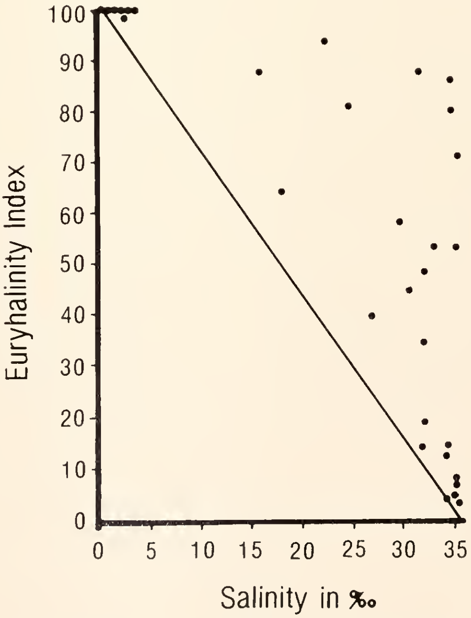

FIGURE 2. Weighted euryhalinity indices for *Callinectes* spp. at 31 Jamaican sites. The diagonal line estimates the euryhalinity indices of hypothetical sites with unvarying salinities.

Mean salinity and $\log_{10}$ (100-EI) are very highly correlated ($r = 0.926$ with 29 df; $P << 0.001$). Hence, 86% of the variation in EIs is explained by salinity. But what causes the increasing variability in untransformed EIs with increasing mean salinity? Apparently it is differential likelihood of severe dilution among sites having equal modal salinities.

Likelihood of severe dilution can be estimated without circularity (in this case, without using knowledge of portunids' tolerances) by using the previously discussed criteria (degree of enclosure, depth and distance from shore), non-portunid indicator species and published records. Jackson (1972) found rather constant salinities in eastern Discovery Bay (Site 16), while Taylor, Seliger, Fastie and McElroy (1966) found highly variable salinities in eastern Falmouth Bay (Site 9). I ranked the 11 Jamaican marine sites in Figure 2: Site 12 (the least variable, 1); 1 = 5 = 6 = 13 = 16 (all 4); 4 = 21 (both 7.5); 3 (9); 31 (10) and 9 (the most variable, 11). These ranks are highly correlated with ranked EIs (Spearman's $r_s = 0.808$; $P < 0.005$). Thus, in low salinities, variance among euryhalinity indices is small because conditions are constantly harsh, *i.e.,* always severely diluted. As usual conditions become less harsh, the variance among sites increases because EIs increasingly reflect likelihood of severe, rather than modal weather.

Similar patterns occur in regions lacking permanent hyposaline biotopes. In Curaçao, in one of the Schottegat's back bays (just W of Rijkseenheid Boulevard, N of Fokker Weg) about 3.3 km from the Caribbean, the estimated species composition was *C. maracaiboensis* (0.45), *C. exasperatus* (0.45) and *C. bocourti* (0.10). Using physiological data from Jamaican crabs, this assemblage can be estimated to have a euryhalinity index of 94.6. In the Waaigat, a blind channel communicating with the sea about 750 m distant through the narrow mouth of the Schottegat, the estimated species composition was *C. danae* (0.65), *C. exasperatus* (0.20), *C. marginatus* (0.13) and *C. ornatus* (0.02) (EI = 51.0). In the Caribbean at the Avila Beach Hotel, only *C. marginatus* was taken or observed (EI = 6.9). A line in Figure 2, on or above which are EIs for all Jamaican sites, estimates the salinities of hypothetical, climatically unvarying sites having equivalent EIs. The typically marine, but dilution-prone Schottegat back bay has a species composition that would be expected in a constant 2‰, while the progressively less variable Waaigat and Avila Beach Hotel sites have assemblages that would be expected in constant salinities of 17 and 33‰, respectively. Observations from the semiarid area near Santa Marta, Colombia present much the same picture. Thus, in biotopes lacking permanent freshwater input, the euryhalinity of the demersal crab guild is inversely proportional to the amount of exchange with the sea.

Boesch (1977) also found that benthic infauna in the poikilohaline Brisbane River, Australia, are limited by minimum salinities, which is not surprising considering their limited motility. But the findings above imply that swimming crabs do not move in response to changes in weather, which is surprising in view of vigorous escape movements and spawning migrations in various species. Since other fully aquatic decapods select salinities in experimental haloclines (Keiser and Aldrich, 1976), and *C. sapidus* has similar behaviors (along thermal gradients; Hoberg, 1975), Caribbean portunids would seem to have the kinds of behaviors necessary to track suitable salinities. Indeed, one observation shows that *Callinectes* do move in response to changes in weather.

A small Jamaican stream, the Laughlands Little River, was flowing strongly in May (before quantitative sampling began) and early June, 1972; bottom salinities were $1\frac{1}{2}$, 2, and 0‰ during three visits. *C. maracaiboensis, C. bocourti* and *C. sapidus* were active in the stream. From late June through September, flow was much reduced, and bottom salinities were 23 to 33‰. Traps produced not only the freshwater species, but *C. exasperatus* and *C. marginatus,* which had invaded the stream from more saline areas. In this stream, even during a rainstorm, equable salinities would be only a few meters to seaward, but in most other biotopes sampled in this study, crabs would have to travel hundreds or thousands of meters to tolerable salinities, increasing the chance that they would be "trapped" while escaping.

Existing data showing that *Callinectes* both remain in, and leave environments during short-term salinity changes are not contradictory, but are a function of distance from waters with more stable salinity regimes. Since crabs escaping from low salinities can reinvade when salinities increase, but crabs which fail to cannot, the density of potential reinvaders in adjacent equable waters should decrease as the size of the stressed patch increases, decreasing the reinvasion rate. This rate should also decrease with increasing patch size because immigration is proportional to perimeter size, which increases less rapidly than area. In other words, populations of less euryhaline crabs invading increasingly large or remote, temporarily habitable biotopes should approach carrying capacity more slowly, because these biotopes are more difficult to leave and enter.

The high correlation between climatic variability and EI among sites with marine modal salinities is understandable if recolonization is not immediate. After severe dilution differentially defaunates a biotope, reestablishment of a later secondary successional stage (*sensu* Horn, 1974) requires a predictable amount of time unless another disturbance sets the process back. Climatically stable sites have sufficient disturbance-free intervals to develop climax community structures, but disturbance-prone sites are temporally dominated by disequilibrium species. This agrees with conclusions in a recent study of temperate infauna by McCall (1977).

The similar salinity zonation patterns throughout the Tropical West Atlantic imply consistent ranking of species' hyposalinity tolerances, but substantial quantitative differences were found between hyposalinity tolerances of Jamaican and Florida Keys *C. ornatus.* One possible explanation concerns differences in test conditions. The most likely are the slightly higher salinities in Keys experiments. Differing long-term acclimation effects offer another possible explanation. Keys crabs came from passes between islands, which have somewhat variable weather due to tidal alternation between Florida Bay and Atlantic water masses. A third possibility is that of genetic differences. Either the most stenohaline individuals are selected out from cohorts settling in the Keys (if Caribbean *C. ornatus* are panmictic), or Keys crabs are sufficiently genetically isolated to permit adaptation to local conditions. Differences in osmoregulatory ability have been shown in two European *Carcinus maenas* (Linnaeus) populations (Theede, 1969), but we need to learn something of population genetics and larval dispersal in *Callinectes* before these differences can be explained.

I dedicate this paper to Harriett Albert Sigman, my mother, whose legacy was the love of the sea and its living things. Many people provided intellectual and logistical support, particularly Gerald Bakus, Heather Copland, Hugh Dingle, Virginia Fox-Norse, John Garth, Alan Havens, Joseph Hegmann, Paul Sammarco and Avril Siung. Virginia Fox-Norse, John Garth, Gordon Gunter, Ingvar Kristensen, William Stephenson and Robert Whittaker critically read an earlier draft. Partial funding came from the University of Southern California's Allan Hancock Foundation, Organization for Tropical Studies, Society of Sigma Xi, American Museum of Natural History's Theodore Roosevelt Memorial Fund, NSF grant BMS 74–22718 to Hugh Dingle and NIMH grant 5 TO1 10641–10 to Neurobehavioral Sciences Program, University of Iowa. This is contribution #156 of Discovery Bay Marine Laboratory.

SUMMARY

This study examines ecological distributions in a guild of Caribbean demersal crabs (family Portunidae) on a gradient in terrestrial influence on aquatic climate, along which the major monotonic physicochemical variable is salinity. Distributions were established by sampling in fresh lotic waters and bays with highly restricted exchange with the sea, which, for marine groups, are constantly and unpredictably climatically severe, respectively, through climatically equable waters around coral reefs. The 16 demersal portunid species collected in Jamaica (the main study area), the Florida Keys, Colombia and Curaçao all occur in undiluted seawater, but progressively fewer are found as salinity decreases. Hyposaline biotopes are virtually monopolized by members of the genus *Callinectes*, while *Arenaeus, Portunus* and *Cronius* spp. were found only in higher salinities. *Callinectes* spp. display serial replacement along the gradient; crab stages of *C. maracaiboensis, C. bocourti* and *C. sapidus* occur mainly in fresh waters, while dominance peaks occur in progressively higher salinities for *C. exasperatus, C. danae, C. marginatus* and *C. ornatus*. Acute hyposalinity tolerances of the common species were determined experimentally, and follow the same order as upstress limits and dominance peaks. The species composition of the guild changes from domination by the most to the least euryhaline species as likelihood of severe dilution decreases. In biotopes with temporarily ameliorated weather, less euryhaline species invade and may replace more euryhaline species at a rate proportional to the biotope's remoteness from the immigrants' source area. Freshwater *Callinectes* are catadromous, storing energy in hyposaline ecosystems, but reproducing in higher salinities.

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POSITIVE AND HOST-INDUCED NEGATIVE PHOTOTAXIS OF THE SYMBIOTIC WATER MITE *UNIONICOLA FORMOSA*

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Many animals respond to certain environmental parameters by an array of taxes and kineses. The initiation and maintenance of numerous symbiotic associations often involve such behavioral responses mediated by one or more sensory modality (Davenport, 1955, 1966). The symbiosis of the freshwater mite *Unionicola formosa* (Acarina: Unionicolidae) and the mussel *Anodonta imbecilis* (Schizodonta: Unionidae) includes several components potentially involving behavioral interactions between symbiont and host.

Female specimens of *U. formosa* deposit eggs in the gills of the host mussel. Larvae emerge in the spring, leaving the host and possibly becoming parasitic on chironomids (Jones, 1965; Böttger, 1976). They subsequently reinvade a mussel and embed in the gills, forming a quiescent transformational stage, the nymphochrysalis, from which emerges the sexually immature nymph. Whether nymphal *U. formosa* leave the host remains uncertain, but ultimately they enter host gills and form the quiescent teleiochrysalis from which the mature adult emerges. As many as 70 mature female mites may occupy the host mussel's mantle cavity, but rarely more than a single male (Roberts, 1977).

The first experimental analysis of a symbiosis involving unionicolid mites was that of Welsh (1930) who reported that *U. ypsilophora* was positively phototactic when washed free of any chemical influence of its host, *Anodonta cataracta,* but displayed negative phototaxis when tested in water containing host gill homogenate or in water from the mantle cavity of the mussel. Welsh (1930) contended that this negative phototaxis helped keep mites in the host's mantle cavity. Reversal of the mite's phototaxis was species-specific in that only tissue homogenate from the species of mussel with which mites had been associated in the field elicited the response (Welsh, 1931). Subsequent study (Welsh, 1932) revealed a positive photokinesis by *U. ypsilophora,* a behavioral response later employed by Waterman (1937) to determine the spectral sensitivity of this organism. To our knowledge no study to date has either characterized more fully the positive phototaxis of unionicolid mites or examined the host-induced negative phototaxis.

Preliminary experiments revealed that *U. formosa* is positively phototactic when tested in water which is free of any host influence and negatively phototactic in the presence of some chemical signal of host origin. This reversal of phototaxis occurs in males, females, nymphs and perhaps larvae. This paper examines the spectral and intensity sensitivity characteristics for both positive and host-induced negative phototaxis of the readily obtainable adult female mites. It is likely that the *U. ypsilophora* of Welsh (1930, 1931, 1932) and Waterman (1937) is in reality *U. formosa,* since the two forms are similar and *U. ypsilophora* apparently does not occur in North America (Mitchell, 1957).

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MATERIALS AND METHODS

Anodonta imbecilis with *Unionicola formosa* was collected from November 1976 to May 1977 from Par Pond, Aiken County, South Carolina. Adult female mites were removed from their hosts and held 1 to 10 days in artificial pond water (APW) (Prosser, 1973), which was changed daily and immediately before each series of experiments. Preliminary experiments revealed that phototactic behavior was unchanged within this time period. All animals were kept at 18 to 20° C under naturally occurring illumination from laboratory windows. Experiments were always performed between 1000 and 1600 hr.

Measurements of phototaxis were conducted in a clear lucite chamber (119 × 20 × 20 mm) which was provided with removable partitions that subdivided the chamber into five equal compartments. The chamber could be illuminated horizontally from one end with a Leitz slide projector (300 W tungsten filament bulb) to which was attached a filter holder. The light was filtered first through an infrared absorbing filter (Corning No. 1-75) and a "hot mirror" (Baird Atomic) to reduce the radiation above 700 nm. The light was then modified by the interposition of thin film filters (3-cavity; halfband pass 7 to 8 nm; Ditric Optics Co.) to control wavelength, and neutral density filters (Ditric Optics Co.) to control intensity. Light intensity was measured with a quantum sensor (Lambda Instrument Corporation Model LI-185).

The test medium in the chamber was either APW or APW to which had been added homogenate prepared as follows: mantle tissue was excised from healthy mussels and ground in cold APW in a tissue homogenizer, diluted with APW to 1.0 g wet weight tissue/10 ml APW, filtered through a 0.45 μ Millipore filter and frozen until used (no more than 5 days after preparation). Immediately prior to use the homogenate was thawed, refiltered, and diluted to a final concentration in the chamber of 0.02 g wet weight tissue/ml APW. Mantle tissue was selected for the homogenate, since it was never used by *U. formosa* as a site of egg deposition and only rarely for the transformational forms.

Mites were light-adapted for at least 1 hr under incandescent room lights plus a 60 W incandescent bulb or dark-adapted 1 hr or more. In tests using dark-adapted animals the mites were transferred to the test chamber under low intensity red light from a GE 25 W red incandescent bulb which was wrapped with several layers of red cellophane. All tests were performed at 20 to 22° C.

The experimental protocol consisted of placing 30 to 35 mites into the central compartment of the chamber to which the desired test medium had been added. Preliminary experiments revealed no significant interaction among mites at this density. The animals were held 30 sec in the dark following introduction into the test chamber. The partitions were then removed and the monochromatic test light turned on. Mites tested for positive phototaxis (no host homogenate present) were stimulated for 60 sec, while those tested under conditions eliciting negative phototaxis (host homogenate present) were exposed to the light for 90 sec, since negative phototaxis took longer to be expressed. At the end of each test the partitions were reinserted and the number of mites in each section was recorded.

Two series of experiments were conducted. Spectral sensitivity was established for light- and dark-adapted mites by determining phototactic responsiveness upon stimulation with approximately equal quantal intensities of the test wavelengths. The different responsiveness of light- and dark-adapted mites during positive and negative phototaxis necessitated using several quantal intensities. Intensity sensitivity was examined by monitoring phototactic responsiveness to a range of intensities of 500 nm light.

The data are presented as percent response based on the number of mites in the section of the chamber closest to the light source (positive phototaxis) or the section farthest from the light source (negative phototaxis). Where appropriate, the data have been analyzed by the Kolmogorov-Smirnov goodness of fit test (Zar, 1974), which compared the distribution of mites in the five sections of the chamber under various test conditions. Control data were generated by repeating the experimental procedure without the stimulus light and monitoring the distribution of mites in the chamber.

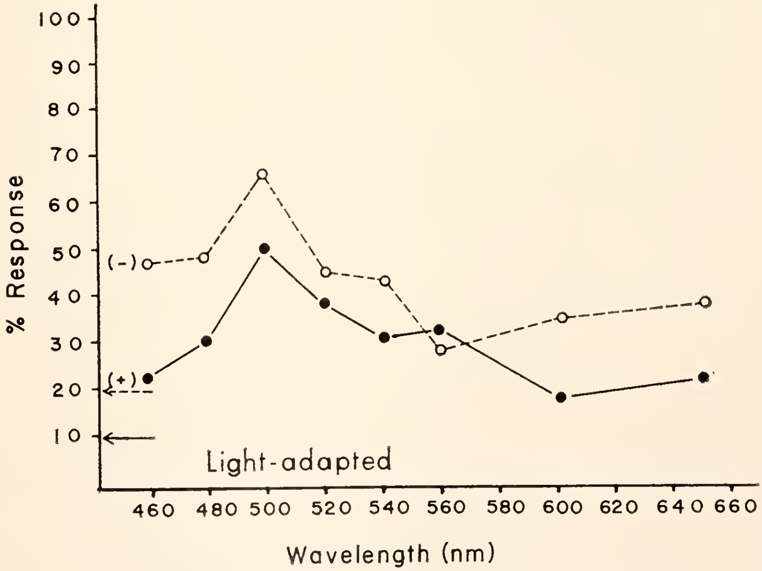

FIGURE 1. Spectral sensitivity curves of positive (solid line) and negative (dashed line) phototaxis of light-adapted *U. formosa.* Positive phototaxis was generated in clean artificial pond water (APW), and negative phototaxis was in APW plus host homogenate. Mean intensities (quanta/m^2/sec) for positive and negative phototaxis are $3.210 \times 10^{12} \pm 8\%$ and $2.248 \times 10^{15} \pm 10\%$, respectively. Arrows indicate the control levels. The N for each condition ranged from 62 to 69.

RESULTS

Spectral sensitivity curves for positive and negative phototaxis of light-adapted female *U. formosa* are depicted in Figure 1. The magnitide of both the positive and negative responses was influenced by intensity (as revealed by other experiments), but the patterns expressed all were similar to those of Figure 1, with maximal sensitivity for both positive and negative phototaxis occurring around 500 nm. The positive phototaxis to 500 and 520 nm (Fig. 1) was significantly greater ($P < 0.05$, Kolmogorov-Smirnov) than that of controls or that associated with any other wavelength. Negative phototaxis was significantly greater ($P < 0.05$) at 500 nm than at any other wavelength.

Spectral sensitivity curves for dark-adapted animals are presented in Figure 2. Positive phototaxis had a less well-defined maximum for dark adapted mites and continued into longer wavelengths than did the positive response of light-adapted animals (Fig. 1). For example, positive respones at 500 to 560 nm were not different from each other, but were significantly different from controls and from responses at other wavelengths. Dark-adapted mites were maximally negatively phototactic at wavelengths of 460 to 500 nm. The response at 480 nm was significantly higher ($P < 0.05$) than at any other wavelength except 460 nm. Dark-adaptation also decreased the sensitivity of negatively phototactic mites to wavelengths longer than 500 nm. It is unclear why the patterns of positive and negative phototaxis agree for light-adapted animals (Fig. 1), but are dissimilar for dark-adapted mites (Fig. 2). However, the responses of dark-adapted

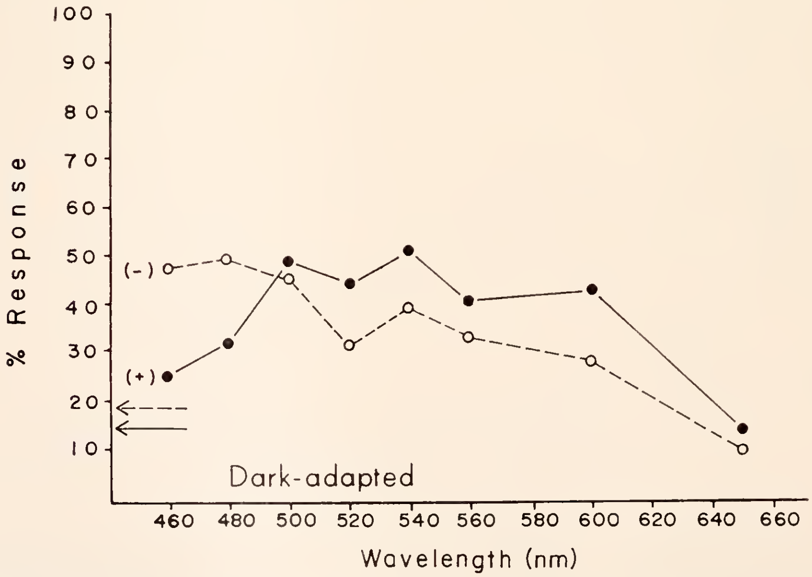

FIGURE 2. Spectral sensitivity curves of positive (solid line) and negative (dashed line) phototaxis of dark-adapted mites. Positive phototaxis was generated in APW, while negative phototaxis was in APW plus host homogenate. Mean intensities (quanta/m²/sec) for positive and negative responses are 3.089×10^{11} ±7% and 3.105×10^{14} ±7%, respectively. N = 87–103. Arrows indicate controls.

animals probably represent the truer spectral sensitivity curves. Since 500 nm light evoked significant responses during all spectral sensitivity tests, this wavelength was used to determine the phototactic responses of mites to different intensities.

Figure 3 depicts the intensity-dependent phototaxis to 500 nm of light- and dark-adapted animals tested in APW containing no host homogenate. No significant ($P > 0.05$) negative phototaxis occurred in any experiment in which host homogenate was absent from the medium. Dark-adaptation increased the intensity sensitivity of *U. formosa,* resulting in a shift to lower intensities of both maximal phototaxis (from between 1.5×10^{-2} and 1.5×10^{-4} μEinsteins to between 1.5×10^{-3} and 1.5×10^{-6} μE) and the threshold for positive phototaxis (from 1.5×10^{-5} to 1.5×10^{-6} μE). (Note that 1.5×10^{-6} μE of 500 nm light $\cong 3.6 \times 10^{-5}$ μW/cm^2.)

The phototactic sign of animals tested in medium containing host homogenate was a function of intensity (Fig. 4), as the animals were negative to high intensities and positive to low, except for dark-adapted animals at very high intensity (Fig. 4B). The occurrence of negative phototaxis at high intensities (Fig. 4A) implies that the higher the perceived light intensity, the greater the negative response. Thus, it is appropriate to measure the negative response in the determination of spectral sensitivity (Figs. 1, 2). It is unknown why the shift in phototactic sign was less well-defined in dark-adapted animals (Fig. 4B) than in light-adapted mites (Fig. 4A). Dark-adaptation resulted in a shift to lower intensities for maximal negative phototaxis (from 10–10^{-1} μE) and for the point of transition between negative and positive phototaxis (from between 10 and 1 μE to approximately 7.0×10^{-5} μE).

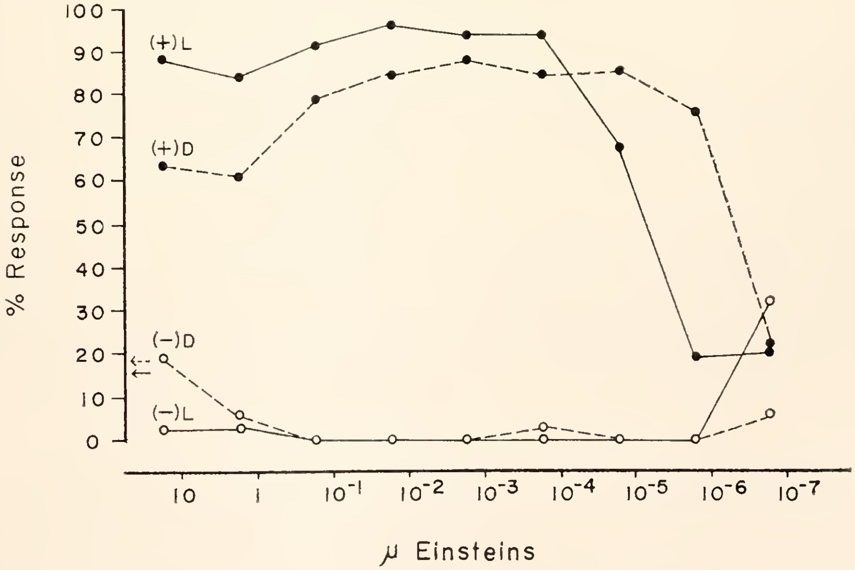

FIGURE 3. Effect of intensity on phototaxis of *U. formosa.* Positive (solid circles) and negative (open circles) phototaxis of both light- (solid lines) and dark-adapted (dashed lines) mites were tested at varying intensities of 500-nm light. All tests were in clean APW. N = 30–36. Arrows indicate controls.

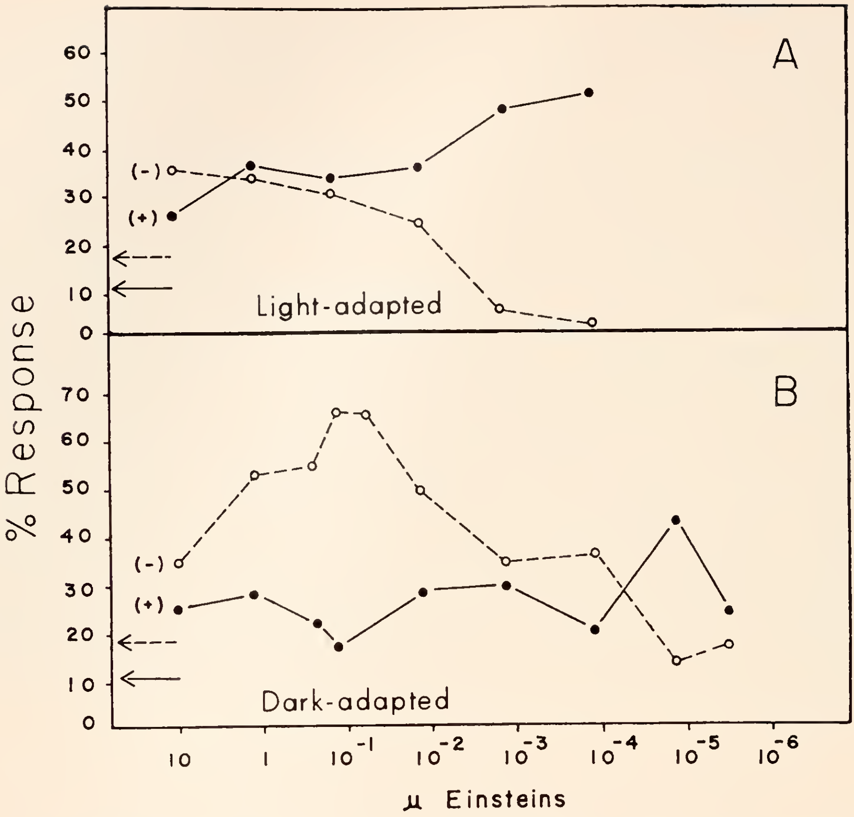

FIGURE 4. Effect of intensity of 500-nm light on phototaxis of light- (A) and dark-adapted (B) mites. Positive (solid line) and negative (dashed line) phototaxis were generated in media containing host homogenate. N = 63 — 68 (A) and 32 — 34 (B). Arrows indicate controls.

Positive and negative phototactic responses of both light- and dark-adapted animals exposed to various wavelengths in APW with host homogenate are depicted in Figure 5. These data are representative of the pattern observed at several quantal intensities. Mites were consistently negatively phototactic at shorter wavelengths but became increasingly positively phototactic at longer wavelengths, a phenomenon particularly evident among light-adapted animals (Fig. 5A).

DISCUSSION

Unionicola formosa exhibited only positive phototaxis when tested in pure APW. In the presence of mantle tissue homogenate from its host, *Anodonta imbecilis,* the sign of the mite's phototaxis was negative to high light intensities. This confirms the work of Welsh (1930).

Maximal sensitivity for positive and negative phototaxis of light-adapted mites occurred at 500 nm. Dark-adapted animals were maximally positively phototactic

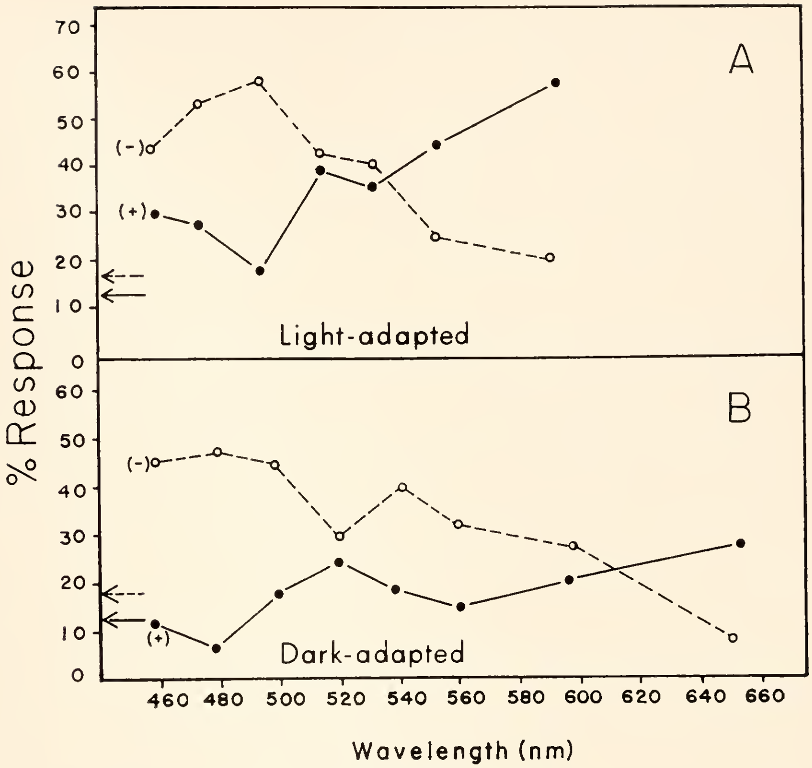

FIGURE 5. Effect of wavelength on the sign of phototaxis of light- (A) and dark-adapted (B) *U. formosa.* Both positive (solid line) and negative (dashed line) phototaxis were generated in test media containing host homogenate. Mean intensity (quanta/m^2/sec) is 2.132 $\times 10^{18}$ ± 16% (A) and 3.105 $\times 10^{14}$ ± 7% (B). N = 32 − 35 and 92 − 103 for A and B, respectively. Arrows indicate controls.

between 500 to 540 nm, while their negative phototaxis was maximal from 460 to 500 nm. Because dark-adapted animals yield data which are more accurate measures of true sensitivity, it is difficult to define a spectral sensitivity maximum. Waterman (1937), working only with the positive response, found that the velocity of crawling toward a light source by partially light-adapted *U. ypsilophora* (*U. formosa*) was maximal at 430 nm, with lower magnitude responses at 485, 575, and 595 nm. Thus, the spectral sensitivity of *U. formosa* is similar to that of numerous planktonic organisms (Forward, 1976a) and benthic species such as the crayfish *Procambarus clarkii* (Hanaoka, Suganuma, Ikari, and Yasumi, 1957).

Many larval aquatic arthropods are positively phototactic at moderate or low light intensity and photonegative at higher intensities (Thorson, 1964), but positive responsiveness to moderate intensity and avoidance of low intensity light has also been observed (Forward, 1974, 1976b, 1977). No change from positive to negative phototaxis by *U. formosa* occurred in clean APW upon stimulation with the experimental light intensities. However, in the presence of host tissue homogenate, negative phototaxis was the dominant response to higher intensities while positive phototaxis predominated at lower intensity levels (Fig. 4), a pattern much more pronounced among light-adapted mites (Fig. 4A). The relatively low responsiveness of dark-adapted mites to very high intensities probably was due to overstimulation of the visual system. This is suggested by the observation that dark-adapted mites became disoriented and randomly active in the experimental chamber upon exposure to high intensity stimulation. Dark-adaptation increased the intensity sensitivity of *U. formosa* (Fig. 3), resulting in a threshold for positive phototaxis of about $1.5 \times 10^{-6}\ \mu E$ ($\cong 3.6 \times 10^{-5}\ \mu\ W/cm^2$), a value similar to those of other aquatic arthropods (Forward, 1976a).

The patterns of the response spectra of *U. formosa* invite speculation concerning the characteristics of this organism's visual pigment(s). The breadth of the response spectrum for positive phototaxis could result from a single pigment which absorbs strongly over a broad range of wavelengths, a phenomenon not uncommon for invertebrate visual pigments (Wasserman, 1974). Alternatively, multiple pigments could be involved (Waterman, Fernandez, and Goldsmith, 1969). The differences with respect to wavelentgh of the positive and negative phototaxis of *U. formosa* (Fig. 2) suggest the presence of two separate pigments, one absorbing maximally between 460 to 500 nm and responsible for negative phototaxis, the other absorbing maximally in the longer wavelengths and involving positive phototaxis (Fig. 5). Why this change in phototaxis is more pronounced in light- than in dark-adapted animals is unknown. However, the observed differences perhaps can be explained as effects of intensity rather than by invoking the presence of a dual pigment system. The varied response of *U. formosa* tested with 500 nm light in the presence of host homogenate, *i.e.,* negative phototaxis to high and positive to low intensities (Fig. 4), suggests that longer wavelengths may be perceived as of low intensity, to which the more sensitive positive response occurs, while shorter wavelengths may be perceived as of higher intensity, eliciting the negative phototaxis. Thus, "wavelength-dependent" phototaxis may be an intensity effect.

The functional significance of this behavior in *U. formosa* is not completely understood. It has been hypothesized that the chemically mediated reversal of phototaxis serves as a mechanism by which mites remain inside the mantle cavity of a host mussel (Welsh, 1932). Alternatively, negative phototaxis upon exposure to some 'host factor' in the water column could serve to bring specimens of *U. formosa* that are in free-living stages into closer proximity of a benthic potential host mussel. Subsequent analysis may reveal that the observed behavior of adult mites is a retention of larval or nymphal behavior.

Summary

1. The symbiotic mite *Unionicola formosa* is positively phototactic when free of any chemical influence of its molluscan host, *Anodonta imbecilis,* and negatively phototactic in the presence of host tissue homogenate.

2. Light-adapted mites exhibit maximal positive and negative phototaxis at 500 nm, while dark-adapted animals display maximal positive phototaxis between 500 and 540 nm and greatest negative responsiveness between 460 and 500 nm.

3. Dark-adaptation increases the sensitivity of *U. formosa,* resulting in a 1 to 2 log unit shift to lower intensities for maximal phototaxis and of the thresholds for both positive and negative responses.

4. *Unionicola formosa* tested in host homogenate exhibits negative phototaxis at high intensities and postive phototaxis at low intensities.

5. An apparent wavelength-dependent change in phototactic sign is more likely a function of differential sensitivity to intensity.

6. Although presumably a means by which host-symbiont contact is enhanced, the functional significance of the host-influenced behavior of *U. formosa* remains speculative.

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CELLULAR MORPHOLOGY AND ARCHITECTURE DURING EARLY MORPHOGENESIS OF THE ASCIDIAN EGG: AN SEM STUDY

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Classical studies of ascidian embryogenesis, especially Conklin's (1905) excellent description, have established that ascidian gastrulation begins during seventh cleavage (see Berrill, 1955, Table II). That is, the most dramatic changes in cellular morphology and architecture occur in the embryo composed of about 100 to 200 cells. This number is quite small, compared to that of an early gastrula of other animals; the early gastrula of sea urchins consists of about 800 cells (Hinegardner, 1967), and the number of constituent cells of an amphibian gastrula is about 13,000 (Hara, 1977).

Scanning electron microscopy (SEM) is a powerful method of accurately and efficiently representing the complex three-dimensional morphology of rapidly growing embryonic systems (Waterman, 1972). Not only does SEM provide abundant opportunities for visualizing the surface features of developing embryos, but also observations with SEM of cut or fractured surfaces can provide information regarding the cellular architecture of embryos and the internal surface topography of hollow structure. During the past few years, several workers, utilizing SEM, have analysed morphological changes of the cell surface during gastrulation in a variety of animals (Monroy, Baccetti and Denis-Donini, 1976; Keller and Schoenwolf, 1977; Turner and Mahowald, 1977). The present paper deals with cellular morphology, contact and arrangement in the blastula, gastrula and neurula stages of the ascidian, *Halocynthia roretzi*, as examined by SEM. The observations have emphasized several characteristic features of the early morphogenesis of the ascidian egg.

Materials and Methods

Naturally spawned eggs of the ascidian, *Halocynthia roretzi*, were reared in filtered sea water at room temperature (15–16° C). The embryos were fixed either in 2.0% glutaraldehyde in 0.1 M cacodylate in sea water (pH, 8.1) or in 1.0% osmium tetroxide in 0.1 M cacodylate in sea water, and rinsed in the buffered sea water. After removal of the chorion, the embryos were dehydrated through a graded series of ethanols. To reveal internal details of the embryos, selected stages were dissected in the appropriate plane with fine forceps while they were in 70% ethanol. All specimens were dried by the critical point technique, using CO_2 with amyl acetate as the transition fluid, with a Hitachi HCP-1 apparatus. The dried embryos were mounted on aluminum stubs, coated with a thin layer of gold utilizing a Giko IB-3 ion coater, and examined with a Hitachi S-310 SEM.

In order to supplement and substantiate the results of the examination with SEM, attentive observations on living materials were also carried out.

RESULTS AND OBSERVATIONS

The 64-cell stage

The outer surface of the embryo examined with the SEM was essentially smooth (Figs. 2 and 3). For a while after cell division, thin cytoplamic processes like microvilli could be seen on the cell surface bordering the interstices between the daughter cells. The two or three polar bodies were present near the animal pole (Fig. 2).

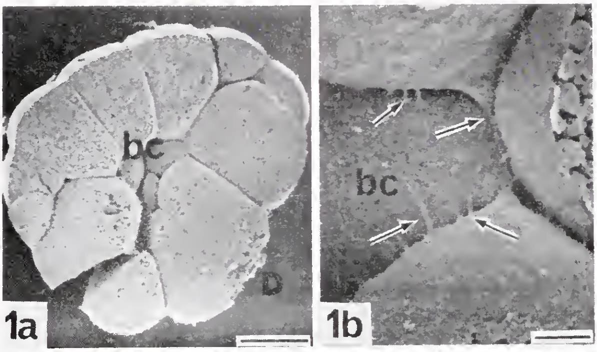
Examinations of the dissected embryos revealed the formation of the blastocoel, which was enclosed by a layer of cells (Fig. 1). The cells of both hemispheres were of nearly equal height at this stage, those of the vegetal (dorsal) hemisphere being slightly taller than those of the animal (ventral) hemisphere (Fig. 1a). On the cell surface bordering the blastocoel numerous cell processes were extended from cells onto adjacent cells (Fig. 1b). The cell processes or filopodia were about 0.2 to 0.5 μm in diameter and varied from 2 μm to over 10 μm in length (Fig. 1b). The blastocoel was observed for a while after cell division. By the time the divided cells adhered closely together, the blastocoel had disappeared.

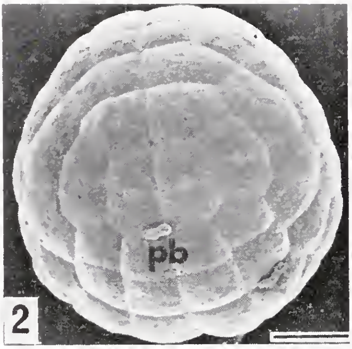
Gastrulation

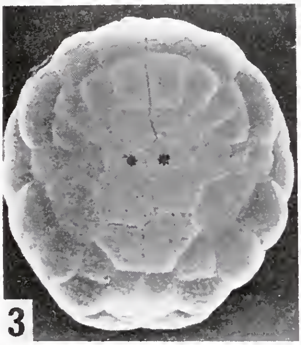
In the egg of *H. roretzi*, similar to those of other ascidians, gastrulation began during the seventh cleavage. The seventh cleavage began on the anterior part of the dorsal (vegetal) hemisphere. After a while, divisions of all the cells of the ventral (animal) hemisphere took place synchronously. After these divisions, changes in cell shape and cellular architecture of the embryo progressed rapidly. The outer surface of the dorsal cells became smaller than that of the vegetal ones; the meso- and endoderm cells at the dorsal hemisphere grew slightly long and narrow, while the ectoderm cells at the ventral hemisphere became broad and flat (Figs. 2, 3, 6; also, compare Fig. 6 to Fig. 1). At the same time a layer of the ectoderm cells and that of the meso- and endoderm cells became adhered closely together (Fig. 6). Thereafter, the blastocoel could no longer be observed (Figs. 6–8).

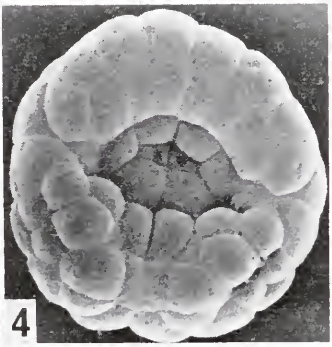
The initiation of gastrulation was first detected by the reduction of the surface area of the two cells of the dorsal hemisphere (Fig. 3). The two cells were $\mathrm{A}^{7\cdot1}$ and $A^{7\cdot1}$ of Conklin's (1905) nomenclature. Examinations of the dissected early gastrula along the midsagittal plane revealed that the two cells changed in shape from tall columnar to wedge-shaped cells (Fig. 6). Their apices became constricted and their bases enlarged (Fig. 6). Viewing from the dorsal pole, the endoderm cells seemed to invaginate, led by the two cells (Fig. 4). In the dissected early gastrula the layers of the ectoderm and endoderm cells adhered closely together, and the ectoderm layer began to fold the endoderm layer (Figs. 6–8). By further epiboly or enfolding movement of the ectoderm cells coinciding with a folding down of the endoderm cells, the gastrocoel was formed (Figs. 8, 11)

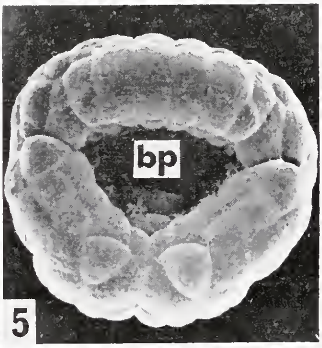
The blastopore of the middle gastrula was of a peculiar form, wide in the anterior and narrow in the posterior (Fig. 5). This might be due to different outgrowths or epibolic movements of the ectoderm cells. The outgrowth began in

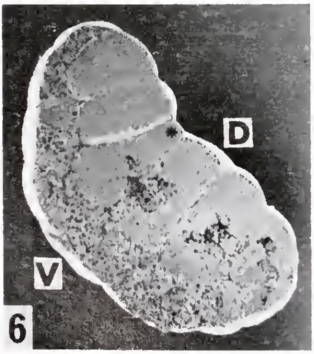


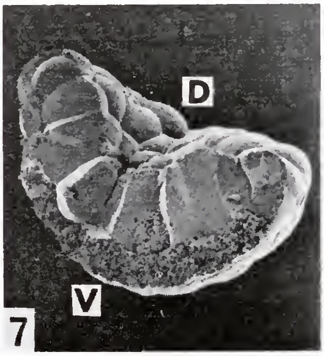












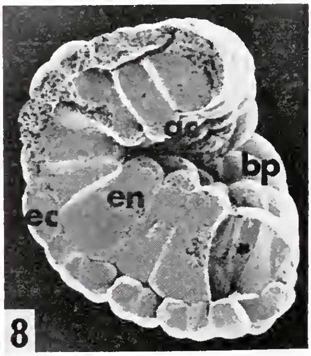


FIGURE 1. Scanning electron micrograph (SEM) of the dissected 64-cell stage showing the formation of the blastocoel (bc). (1a) The cells of both hemispheres are of nearly equal height (D, dorsal side; V, ventral side). Bar, 50 μm. (1b) Cell surface bordering the blastocoel. The cells extend cytoplasmic processes or filopodia onto adjacent cells (arrows). Bar, 5 μm. The embryo is shown with the anterior end up and the posterior end down in all figures.

FIGURE 2. Early gastrula stage viewed from the ventral (animal) pole. The ectoderm cells have divided seven times (pb, polar bodies). Bar, 50 μm.

FIGURE 3. Initial gastrula stage viewed from the dorsal (vegetal) pole. Note the reduction of the surface area of the endoderm cells, especially the two cells marked by asterisks.

FIGURE 4. Early gastrula stage viewed from the dorsal pole. Emboly of the endoderm cells begins. Asterisks show the two leading cells. Several meso- and endoderm cells are dividing.

FIGURE 5. Middle gastrula stage viewed from the dorsal pole. The blastopore (bp) is

the front of the anterior quadrants and progressed rapidly. At the right and left sides of the blastopore the outgrowth was slow, while at the posterior pole the outgrowth was further delayed (Figs. 4, 5). The gastrocoel cavity was wide in the anterior, narrowing towards the posterior (Fig. 8). The cell surface bordering the gastrocoel was generally round and smooth without cell processes (Figs. 8, 11). At the late gastrula stage, the blastopore shifted posteriorly by rapid epibolic movement of the anterior lip (compare Fig. 5 to Fig. 9). During the succeeding phase of gastrulation, the blastopore shifted back from posterior to dorsal by the outgrowth of the posterior lip, as well as that of right and left side lips (compare Fig. 9 to Fig. 10). Coincident with the shift, the blastopore became narrow and oval (Fig. 10). Examinations of the dissected embryos showed that gastrulation was almost accomplished at this stage (Fig. 11).

At the middle gastrula stage the eighth division of the ventral ectoderm cells occurred almost synchronously. The ninth division of the ectoderm cells took place at the late gastrula stage. The middle gastrula was nearly circular in outline viewed from the dorsal or ventral pole (Fig. 5). At the late gastrula stage the embryo became elongated and egg-shaped, the posterior end being somewhat narrower than the anterior (Fig. 10).

The process of closure of the blastopore temporally overlapped in part with that of neurulation.

Neurulation

The neural tube formation of the ascidian embryo progressed in a similar fashion to that of vertebrates (Schroeder, 1970; Karfunkel, 1974). The neural plate cells were distinguishable on the mid-anterior part of the dorsal side of the late gastrula (Figs. 9, 10). They were flat in shape and consisted of several transverse rows of cells. Neural tube formation proceeded from the posterior end to the anterior end (Figs. 12, 14). Cells were heaped in a mass at the anterior end of the neural fold (Fig. 12). The neural folds of the lateral edges of the neural plate rolled toward the midline. The neural plate cells underwent a gradual elongation in the early stages of neurulation, and then changed in shape from columnar to wedge-shaped cells, the apices of which became narrow (Fig. 13). When the folds met at the midline, the plate was folded into a tube (Fig. 14). The ectoderm lateral to the neural plate was also carried to the midline, and formed the epidermis covering the neural tube.

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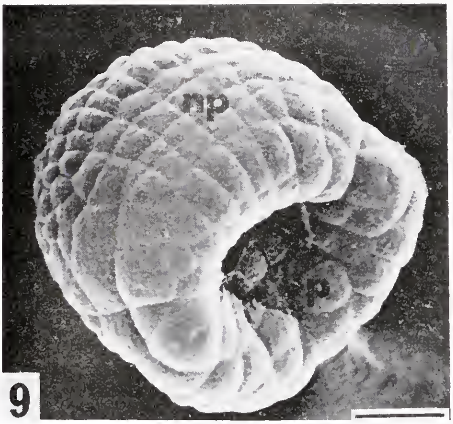
of a peculiar form, wide in the anterior and narrow in the posterior. This may be due to different outgrowths of ectoderm cells.

FIGURE 6. Dissected initial gastrula stage along the midsagittal plane showing that the cells of both hemispheres adhere very closely together. The ectoderm cells of the ventral hemisphere (V) become shorter than the meso- and endoderm cells of the dorsal hemisphere (D). The invaginating cell becomes wedge-shaped (asterisk).

FIGURE 7. Dissected early gastrula stage along the midsagittal plane. The cells of the ventral hemisphere (V) begin to enfold those of the dorsal hemisphere (D).

FIGURE 8. Dissected middle gastrula stage along the midsagittal plane. By further enfolding movement of the ectoderm cells (ec), the gastrocoel (gc) is formed (bp, blastopore; en, endoderm cells).



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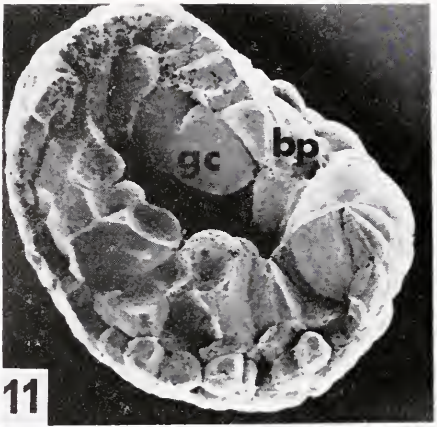

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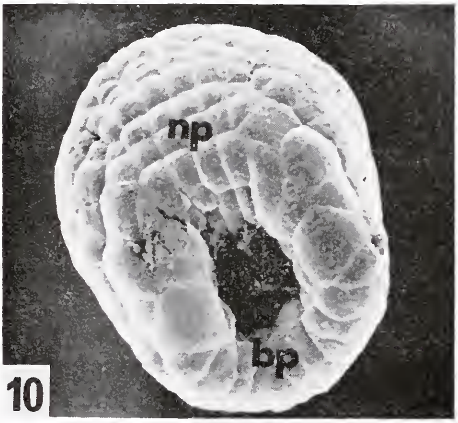

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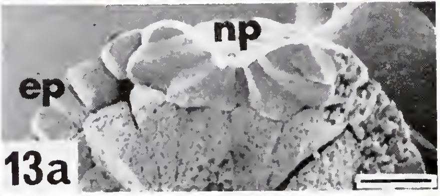

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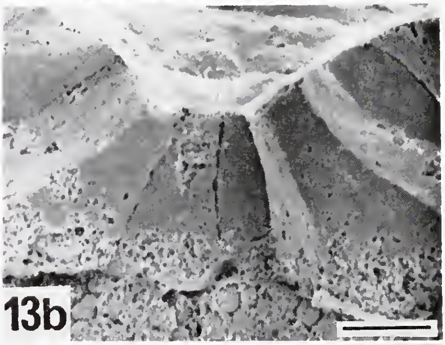

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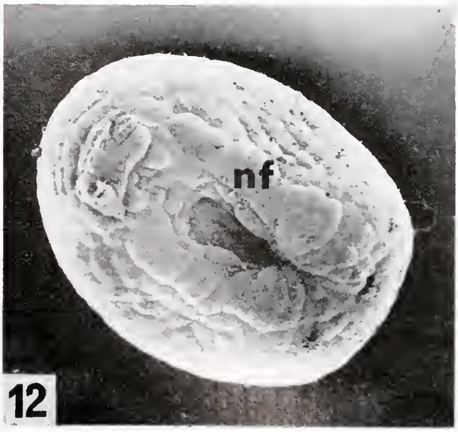

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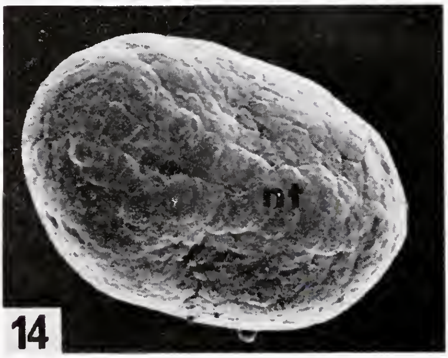

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FIGURE 9. Late gastrula stage viewed from the dorsal side. The blastopore (bp) has shifted from the mid-dorsal portion of the embryo to the posterior portion. The neural plate cells (np) are distinguishable. Bar, 50 μm.

DISCUSSION

Early morphogenesis, including gastrulation and neurulation, of animal eggs is a phenomenon of many integrated cell movements, each of which depends directly or indirectly on every other (Trinkaus, 1969). Morphogenetic movements always involve deformation of constituent cells of the system. Based on accurate observations on living eggs and embryos, Conklin (1905) suggested two major factors for ascidian gastrulation; the first is the change in shape of the cells of the animal and vegetal hemispheres, and the second is the overgrowth of the marginal cells of the animal hemisphere. The present observations with SEM have fairly confirmed these two factors. The change in shape of the cells during gastrulation and the enfolding movement of the ectoderm cells can easily be imagined by comparing SEM photographs of dissected gastrulae. Another feature of the cell architecture during gastrulation of ascidian eggs may be the close adherence of the layer of the ectoderm cells and that of the meso- and endoderm cells. The blastocoel was observed in the dissected blastula for a while after cell division. However, by the time the divided cells adhered closely together, the blastocoel had disappeared. Pseudopodia observed on the cell surface bordering the blastocoel seem to contribute to cell-to-cell adhesion.

Mancuso (1973), utilizing transmission electron microscopy, has observed ultrastructural characteristics of the constituent cells in ascidian gastrula and neurula, and revealed bundles of microtubles at the peripheral, external cytoplasm of the neural blastomeres. At the early gastrula stage the endoderm cells changed in shape from columnar to wedge-shaped cells. Similarly, the neural plate cells changed in shape from flat, through columnar, to wedge-shaped cells. These changes in shape might be caused by a cytoplasmic system acting at the surface of wedge-shaped cells. It is expected that cytoplasmic contracting systems such as microfilaments act at the surface of the wedge-shaped cells.

In conclusion, the present observations with SEM of the ascidian morphogenesis has fairly confirmed the results of descriptive studies of ascidian embryogenesis.

The author wishes to express his gratitude to Dr. T. Numakunai of Asamushi Marine Biological Station of Tohoku University for supplying the materials

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FIGURE 10. Neural plate stage viewed from the dorsal pole. The embryo becomes elongated and egg-shaped. The blastopore (bp) becomes narrow and oval. Neural plate cells (np) are flat in shape and consist of several transverse rows of cells.

FIGURE 11. Dissected neural plate stage along the sagittal plane. Enfolding movement of the ectoderm cells has nearly completed (bp, blastopore; gc, gastrocoel).

FIGURE 12. Middle neurula stage viewed from the dorsal pole. The neural tube formation takes place from the posterior end of the embryo to the anterior end. At the anterior end of the neural fold (nf) cells are heaped in a mass.

FIGURE 13. (13a) Dissected early-to-mid neurula stage along the transverse plane showing rolling-up of the neural plate cells (np) (ep, epidermis). Bar, 30 μm. (13b) Higher magnification of the neural plate cells showing close adherence of the cells to one another. They are wedge-shaped. Bar, 10 μm.

FIGURE 14. Late neurula stage viewed from the dorsal side. The folds of lateral edges of the neural plate meet at the midline and then the plate is folded into tube (nt, neural tube).

and to Dr. M. Yamamoto of Tamano Marine Laboratory of Okayama University for teaching him SEM technique. All observations with the SEM have been carried out at Tamano Marine Laboratory. He thanks Prof. M. Yoshida of Tamano Marine Laboratory for affording him opportunities to utilize their facilities.

This study was supported in part by a Grant-in-Aid from the Ministry of Education of Japan (No. 374232).

SUMMARY

1. Cellular morphology and architecture during early morphogenesis of the ascidian embryo were examined by SEM.

2. The outer surface of the embryo was essentially smooth. The blastocoel could be seen in the dissected blastula. On the cell surface bordering the blastocoel, numerous pseudopodia extended from cells onto adjacent cells. These pseudopodia were suggested to contribute to cell-to-cell adhesion.

3. Before the initiation of gastrulation, a layer of the cells of the animal (ventral) hemisphere and that of the cells of the vegetal (dorsal) hemisphere adhered closely together. The blastocoel could no longer be observed.

4. The gastrulation began during the seventh cleavage. The gastrocoel was formed by a folding of the two layers of the cells. Examinations of the dissected gastrulae suggested two cooperative forces for the gastrulation: first, the epibolic or enfolding movement of the ventral ectoderm cells and secondly, the change in shape of the constituent cells.

5. The neural tube formation progressed in a similar fashion to that of vertebrates.

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RESISTANCE OF A TUNICATE TO FOULING

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Although sessile organisms of several phyla are believed to have defenses against epizooic recruitment (fouling), the ecological consequences of epizooic recruitment have received relatively little attention. In simple ascidians, such as *Ascidia nigra,* (Savigny) extensive fouling could have several detrimental effects: first, obstruction of the siphon apertures could prevent feeding and respiration; secondly, over-topping could reduce feeding efficiency by reducing water movement past the ascidian's siphons; thirdly, epibionts could compete with the ascidian for food; fourth, a heavy load of fouling organisms could also increase sedimentation, which could be detrimental; and finally, increased drag due to epibionts could make an ascidian more likely to be dislodged by water movements. However, fouling may have a beneficial effect by providing camouflage.

A. nigra is a common member of fouling communities in the West Indies and Bermuda. This ascidian is free of epibionts, although sedentary organisms settle and grow on the tests of many other ascidians (Goodbody, 1962; Stoecker, unpublished observations). Although they may survive from 18 to 22 months (Millar, 1971), mature *A. nigra* never are overgrown by other epibenthic species. Goodbody and Gibson (1974) noted that, in Jamaica, adult populations of this species were relatively free of competitors and predators. Goodbody (1962) attributed the lack of epizooic recruitment on *A. nigra* to the sloughing off of the test surface.

The ability of ascidians to concentrate vanadium from seawater is well known but poorly understood (Goodbody, 1974; Carlson, 1975; Carlisle, 1968; Danskin, 1978; Webb, 1939, 1956). Vanadocytes are abundant in the blood of *A. nigra.* Partially-complexed vanadium is localized within vacuoles of vanadocytes (Kustin, Levine, McLeod, and Curby, 1976). These vacuoles, called vanadophores, also contain sulfuric acid (Webb, 1939). Vanadocytes are known to migrate through blood vessels into the test. Many ascidians, including *A. nigra,* have sulfuric acid-filled capsules called bladder cells in their tests. The formation of bladder cells is believed to be associated with the degeneration of vanadocytes (Goodbody, 1974).

Vanadium, except in trace amounts, is a metabolic poison (National Research Council, 1974) and is expected to be toxic to a wide variety of organisms. Similarly, strongly acidic solutions are destructive to most tissues. The present study investigates the role of these two inorganic toxins in the prevention of recruitment of *Ecteinascidia turbinata,* an ascidian, and *Pennaria tiarella,* a hydroid, onto the test of *A. nigra.* Both *E. turbinata* and *P. tiarella* produce larvae throughout the summer and are found in the same habitats as *A. nigra* in Bermuda.

Although the roles of organic allelochemicals are known in some organisms

(Whittaker and Feeny, 1971), inorganic toxins have received little attention. Allelochemicals in marine organisms may be effective against parasites and epibionts (Siebruth, 1968; Siebruth and Conover, 1965; Fenical, 1975), competitors (Jackson, 1977; Jackson and Buss, 1975), and predators (Bakus, 1970; Bakus and Green, 1974).

Many functions for vanadium and vanadocytes in ascidians have been proposed; these include oxygen transport (Carlson, 1975), tunicin synthesis (Endean, 1961), phagocytosis of foreign bodies (Brown and Davies, 1971; Wardrop, 1970), cellular immunity (Anderson, 1971) and production of sulfuric acid for an unknown purpose (Swinehart, Briggs, Halko, and Schroeder, 1974). However, there is no general agreement on the functions of vanadium or vanadocytes in ascidians (Webb, 1956; Carlisle, 1968; Carlson, 1975). The present paper proposes that vanadocytes, at least in some species, play a role in chemical defense against fouling organisms.

Materials and Methods

Collection and maintenance of specimens

During the summer of 1977, *A. nigra* individuals between 4 and 12 cm long were collected from bridge pilings, docks, and coral rubble in the vicinity of the Bermuda Biological Station at St. George's, and near the Bermuda Government Aquarium at Flatts, Bermuda. Specimens were maintained in running seawater tanks at the Bermuda Biological Station at ambient temperature and salinity.

P. tiarella stalks and *E. turbinata* colonies were collected in the same habitats and locations as *A. nigra*. Mature *E. turbinata* colonies release larvae when the colonies are transferred to fresh bowls of seawater. *E. turbinata* larvae were collected and used in experiments within five hours of their release.

P. tiarella stalks were collected in the afternoon. For spawning, three female stalks and one male stalk were placed in 200 ml of filtered (0.45 μm Millipore filter) seawater in a Cambridge bowl. The bowls were placed in the dark at 6 PM. Four hours later, the released eggs were transferred to fresh bowls of filtered seawater. In the morning, the unfertilized eggs were removed from the culture bowls. *P. tiarella* planulae were used in experiments on the second day after spawning.

Chemical analyses and pH determinations

Tests of *A. nigra* individuals were removed and rinsed in seawater. The part of the test where the organism had been attached contains large blood vessels and was discarded. The black surface deposit on the test was collected by brushing the external surface in distilled water, washed, and concentrated by centrifugation (Sorval RC-5 centrifuge, GSA head, 10,000 rpm, 15 min). The surface deposit, test, and soft bodies (animals with the tests removed) were weighed and then dried at 60° C. Dried samples were weighed, digested in hot $HNO_3 : H_2SO_4 : : 2 : 1$ in a reflux system, and analyzed for vanadium with an atomic absorption spectrometer using the method of standard additions (Kustin *et al.*, 1976).

The pH of the tunic and body surfaces was determined with a miniature pH probe (Microelectrodes Inc., Londonderry, N.H.).

Morphological analyses

Thin cross-sectional slices of tunic (less than 1 mm) were taken with razor blades, fixed in three percent glutaraldehyde in seawater, and embedded in araldite 506 epoxy resin. Photomicrographs were taken of unstained slices in the embediment to show the distribution of pigment cells. Fresh slices were also stained with neutral red in seawater and examined microscopically to study the distribution of bladder cells in the test.

Larval settlement experiments

Larval settlement experiments were run in Cambridge bowls containing 200 ml of filtered seawater. Twenty-five to thirty-five larvae of *P. tiarella* or *E. turbinata* were added to each replicate bowl. Larval responses to experimental and control substrates were assessed by counting larvae on each substrate. Larvae were examined under low magnification (30X) using a compound scope and classified as settled, active, inactive (no response to prodding or to the switching on and off of the sub-stage illuminator) or dead (evidence of decay). Stolon length of *E. turbinata* was measured with an ocular micrometer.

In the first set of experiments, the tunic of *A. nigra* was tested for anti-fouling properties. Discs 2 cm in diameter were cut from *A. nigra*'s test; control discs were cut from control agar (3% in seawater) darkened with lampblack to control for color. (Lampblack in agar has no effect on *P. tiarella* or *E. turbinata* larvae). Test discs were alternated with control discs (three each) embedded in a control agar substrate at the bottom of replicate Cambridge bowls. Larval response was scored after 19 to 22 hr.

In the second set of experiments, an aqueous extract of *A. nigra*'s test was assayed for anti-fouling properties. The extract was prepared by mixing pieces of fresh test with an equal weight of distilled water in a Waring blender. The resulting mixture was brought to a boil and then filtered through a Whatman #1 filter. The vanadium content of the extract was 23 ppm. The extract had a pH of 2.0. An aliquot of the extract was neutralized with 1 N NaOH.

Cambridge bowls were prepared with one half of the bottom covered by control agar and the other half covered by extract agar. Agars made with the unneutralized extract would not set unless the agar was first dissolved in distilled water (approximately 10 ml per gram of agar) before the extract was added. Larvae were added to the bowls and after three hours they were scored and counted.

In the third set of experiments, the effects of vanadium alone on *P. tiarella* and *E. turbinata* were tested. Vanadium was added as vanadyl sulfate to agar. Sulfuric acid was added to the control agar to obtain a sulfate ion concentration equivalent to that found in the experimental agar with the highest vanadium concentration. Vanadium and control agars were neutralized with 1 N NaOH. Replicate bowls were prepared for each concentration of vanadium tested. Larvae were scored after 16 hr exposure.

Table I

Distribution of vanadium in Ascidia nigra.

Sample	N	ppm Vanadium ± SD	
		Wet weight basis	Dry weight basis
Without tunic	8	144.81 ± 72.69	—
Tunic	6	6.00 ± 4.49	95.78 ± 69.91
Surface deposit	4	253.32*	1021.92 ± 101.21

* Calculated from ppm V dry weight basis.

RESULTS

Chemical analyses and pH determinations

In *A. nigra,* vanadium is not evenly distributed between the test and the soft body parts (test removed) (Table I). The soft body contains about 145 ppm vanadium (wet weight basis), whereas the test contains only 6 ppm (wet weight basis). Ciereszko, Ciereszko, Harris, and Lane (1963), observed a similar distribution of vanadium in *A. nigra.* However, the black surface deposit brushed from the test's surface has a very high vanadium content of approximately 253 ppm (wet weight basis). Vanadium is concentrated in the black surface layer relative to the test as a whole.

The external surface of the test releases a strong acid when it is even slightly bruised (Table II). This acidity is believed to be due to the release of free sulfuric acid from capsules in the test (Webb, 1939; Andrew, 1961; Goodbody, 1974).

Morphological analyses

Black pigment is concentrated near the surface of *A. nigra*'s test, which is several millimeters thick. The deeper layers of the test are semi-transparent. In sections of the test, black cells are abundant, particularly near the outer surface (Fig. 1). These black test cells are probably the same as the blue cells described by George (1930) and the pigment granules described by Hecht (1918) and Goodbody (1962) in *A. nigra*'s test. Test cells have been described in tests of other ascidians which concentrate vanadium or iron (Stievenart, 1970; Smith, 1970). The test cells in *A. nigra* are believed to be derived from vanadocytes, which migrate from blood vessels into the test matrix, where the vanadocytes turn blue-black and begin to degenerate (Hecht, 1918; Berrill, 1950; Goodbody, 1962;

Table II

Acidity of Ascidia nigra.

Sample	N	pH ± SD
Without tunic	6	7.48 ± 0.13
Tunic	6	1.96 ± 0.62

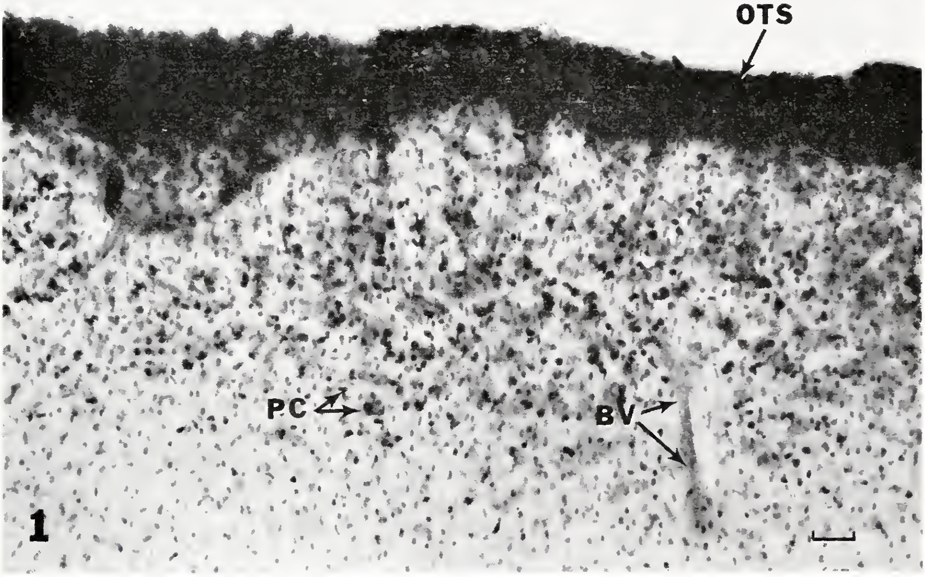

FIGURE 1. Cross-section of *Ascidia nigra* test showing concentration of pigment (black test cells) near outer surface of test. Magnification bar = 100 μ. Abbreviations are: OTS, outer test surface; PC, pigment; and BV, blood vessel.

Smith, 1970). The nuclei of test cells, even those found near the test surface, stain red with Feulgen's stain, indicating that these cells were alive prior to fixation (unpublished observations, D. Stoecker). It is reasonable to propose that the test cells, which are derived from vanadocytes, form the black surface layer described by Goodbody (1974). The derivation of the test cells from vanadocytes would explain the high concentration of vanadium found in the black surface deposit on the test.

Bladder cells, because of their acid contents, stain cherry red with neutral red

TABLE III

Effect of tunic surface on Pennaria *(19–22 hours).*

Treatment	Number of larvae		
	Active	Inactive	Dead
External surface of tunic	1	0	71
Internal surface of tunic	5	34	22
Control	2	21	0

$N = 156$
$G = 124.65$
$P < 0.005$

TABLE IV

Effect of tunic extract on Pennaria (*3 hrs*).

Agar surface	Number of larvae			
	Active	Inactive	Dead	
Extract (pH 2)*	0	11	14	$N = 40$
Control (pH 6)	13	2	0	$G = 41.762$
				$P < 0.005$
Extract (pH 7)*,**	9	3	0	N = 25
Control (pH 6)	13	0	0	G = 1.826
				n.s.

* 22.8 ppm vanadium.
** Neutralized with 1 N NaOH.

in seawater. Bladder cells were abundant in the test but were concentrated just beneath the outer border of the test.

Larval settlement experiments

Table III summarizes the results of several settling experiments designed to assay the response of *P. tiarella* to pieces of *A. nigra*'s test. *P. tiarella* larvae accumulated on test discs relative to control discs. A greater proportion of the *P. tiarella* on test discs than on control discs were inactive or dead. *P. tiarella* larvae probably accumulated on test discs as they were inactivated by the toxic effects of contact with the test.

Contact with the extract agar (pH 2, 23 ppm vanadium) inactivated *P. tiarella* larvae within 3 hr (Table IV). Neutralized extract agar had no significant effect on larvae within the three-hour exposure period (Table IV).

Vanadium had a toxic effect on *P. tiarella* larvae (Table V). Concentrations of 250 ppm (wet weight basis), or more, of vanadium in the substrate significantly

TABLE V

Effect of vanadium on Pennaria (*16 hrs*).

ppm Vanadium	Number of larvae				
	Settled	Active	Inactive	Dead	
0	1	9	13	0	
25	4	7	12	1	
50	2	9	9	1	N = 153
100	3	7	18	2	G = 119.484
250	0	1	30	1	$P < 0.005$
500	0	0	12	11	

A posteriori tests:
ppm Vanadium 0 25 50 100 250 500

Table VI

Effect of tunic surface on Ecteinascidia *(19–22 hours).*

Treatment	Number of larvae			
	Settled	Active	Inactive	Dead
External surface of tunic	4	11	8	6
Control	27	15	11	3

$N = 85$
$G = 12.52$
$P < 0.01$

increased the proportion of *P. tiarella* larvae which were inactive or dead relative to control agar substrates.

E. turbinata larvae, which swim rapidly but erratically until metamorphosis, were not found in greater numbers on the test discs than on the control discs (Table VI). However, a greater proportion of the larvae on test discs were inactive or dead than on control discs.

The extract agar substrate (pH 2, 23 ppm vanadium) rapidly killed *E. turbinata* larvae (Table VII). Neutralized extract agar had no effect on *E. turbinata* within a three-hour exposure period, but within 16 hr a deterimental effect was evident (Table VII).

Concentrations of 250 ppm, or more, of vanadium significantly decreased stolon length in *E. turbinata* (Table VIII). Stolon length should be a good indicator of settlement success.

Table VII

Effect of tunic extract on Ecteinascidia.

Agar surface	Number of larvae		
	Active or Settled	Inactive or Dead	
	(3 hours)		
Extract (pH 2)*	6	27	$n = 64$
Control (pH 6)	25	6	$G = 26.906$
			$P < 0.005$
	(3 hours)		
Extract (pH 7)*,**	20	4	$n = 57$
Control (pH 6)	28	5	$G = 0.024$
			n.s.
	(16 hours)		
Extract (pH 7)*,**	15	16	$n = 48$
Control (pH 6)	14	3	$G = 6.768$
			$P < 0.01$

* 22.8 ppm vanadium.
** Neutralized with 1 N NaOH.

TABLE VIII

Effect of vanadium on Ecteinascidia *metamorphosis (16 hrs).*

ppm Vanadium	N	Mean stolon length (mm)	
0	10	0.53	
50	2	0.44	
100	5	0.52	$F_s = 11.958$
250	5	0.24	$P < 0.001$
500	9	0.20	
1000	2	0.10	

A posteriori tests:

Source of variation	SS	
0 vs 50 vs 100	0.01415	n.s.
250 vs 500 vs 1000	0.02800	n.s.
(0 + 50 + 100) vs (250 + 500 + 1000)	9.63166	$P < 0.001$

DISCUSSION

The results presented here demonstrate that the test of *A. nigra* is toxic to *P. tiarella* and *E. turbinata* larvae. There was no evidence that these larvae avoided the test surface. Similar larval response to a toxic substrate was obtained by Wisely (1962), who found that ectoproct larvae accumulated on surfaces covered with anti-fouling paint even though larvae were killed by contact with the paint. Instances of larval selectivity for settling substrates based on physical factors such as light, temperature, water currents, and contour, texture and angle of the surface are well documented (Osman, 1977). Biological factors, such as presence of a microbial film, type of algae, and presence of the same species, also affect larval settlement (Osman, 1977). However, few data are available on the response of settling larvae to toxic substrates (Wisely, 1962, 1963a, b, 1964).

To understand the toxicity of *A. nigra's* surface, both chemical and morphological characteristics of *A. nigra's* test must be considered. The explanation proposed here is that the vanadium-rich deposit on *A. nigra's* surface is formed by degenerating vanadocytes and prevents epizooic recruitment through its toxicity and by its tendency to slough. The vanadium-agar substrate experiments demonstrated that vanadium alone can inactivate or kill settling larvae. The black surface deposit may partially explain why *A. nigra* is free of epibionts while many other solitary ascidians are fouled.

The external surface of *A. nigra's* test releases a strong acid when it is even slightly injured or bruised. The capsules in the test, which are believed to be the source of the free acid, are concentrated near the test surface. This location would make the capsules effective against organisms which may try to establish a holdfast on the test by penetrating the surface with stolons or other anchoring devices. It is possible that the capsules discharge acid to the test's surface even in the absence of injury (Goodbody, 1974).

Vanadocytes and many of the black test cells are probably alive and may move to sites of injury in the test. The disturbance of the test surface by holdfasts of settling larvae may cause injury. Thus, vanadium and acid may be preferentially released at sites where these toxins are most needed to defend *A. nigra* against fouling and overgrowth.

Not all ascidians which concentrate vanadium or which produce free sulfuric acid are resistant to fouling. For example, *Ascidiella aspersa* concentrates vanadium and has acid-filled test capsules (Webb, 1939; Stievenart, 1970) but lacks typical vanadocytes (Webb, 1939; Berrill, 1950). This species is often covered by epibionts (Berrill, 1950; Stievenart, 1971). Although vanadium is present in this species, there is no evidence that it is concentrated at the test surface. The acid capsules in *A. aspersa* are concentrated in the lower layers of the test (Stievenart, 1970), which may explain why epibionts can attach to the test.

Rhopalaea birkelandi and *Halocynthia aurantium,* like *A. nigra,* are simple ascidians which are resistant to epizooic recruitment (Tokioka, 1971; Smith, 1970). The resistance of these species to fouling has not been investigated. *R. birkelandi* is very acidic (Birkeland, personal communication) and also has a high vanadium content (D. Stoecker, unpublished data). Geranyl hydroquinone has been isolated from *Aplidium* sp. by Fenical (1976), and it is possible that this or other organic toxins, in addition to inorganic toxins, may be involved in chemical defense in some ascidians.

If fouling is often detrimental to epibenthic organisms, one may ask how species which are susceptible to epizooic recruitment avoid the difficulties imposed by fouling. Some ascidians are sexually mature at three weeks, whereas other species may grow for over a year before they are sexually mature (Jackson, 1977). The fast-maturing species may become senescent before fouling organisms interfere with their growth. Therefore, fouling may be inconsequential for these species. Some colonial species, for example *E. turbinata,* periodically die back to stolons and then produce a new generation of zooids from the stolons (Morgan, 1977). *E. turbinata,* although it concentrates vanadium (Ciereszko *et al.,* 1963), is susceptible to fouling (D. Stoecker, unpublished observations). Die-backs may periodically rid colonies of their epibionts.

Vanadium and free acid may be involved in allelochemical interactions between ascidians and their parasites and predators as well as their epibionts and competitors. Extracts of *A. nigra* inhibit the growth of bacteria; Burkholder (1973) believes this effect is due to acidity. Vanadocytes of *Molgula manhattensis* encapsulate large foreign bodies and are involved in graft rejection (Anderson 1971). Anderson has suggested that vanadocytes which invade grafts may rupture and release their vacuolar contents, thus killing the graft. Vanadocytes accumulate at sites of injury (Anderson, 1971; Wardrop, 1970) and may possibly encapsulate or destroy invading organisms.

Newly metamorphosed specimens of *A. nigra,* once they develop the black surface deposit, have low mortality until senescence. Goodbody and Gibson (1974) partially attribute this population stability to lack of predation. The test substance, but not the soft body of *A. nigra,* is distasteful to fish (Goodbody, 1962). Haynes, Sangster, Steven and Thomas (1967) observed that extracts of *A. nigra's* test evoked an avoidance response from fish. Acidity may alone or partially explain the distastefulness of *A. nigra.* Acidic tissues are repellant to most fish (Thompson, 1960). Webb (1939) suggested that the acid-filled capsules in the tests of some ascidians may discourage predator attack. However, some types of ascidians which concentrate vanadium but which are not highly acidic, such as *E. turbinata* and *Clavelina picta,* also seem to be resistant to most general predators (D. Stoecker, unpublished data). Perhaps vanadium or other inorganic or organic chemicals in these ascidians can protect them from predation.

I thank J. S. Levinton, L. B. Slobodkin, I. Duedall, R. G. Summers, and W. E. Sterrer for their valuable advice and assistance. In particular, I thank R. G. Summers for suggesting that I use *Pennaria* and *Ecteinascidia* in the settling experiments and for the histological study of *A. nigra's* test. J. Markham, D. Lonsdale, and personnel at the Bermuda Biological Station helped make this study possible. C. Zeppie and J. Seligman assisted me with the atomic absorption spectrometry. This investigation was supported by a Vollmer Foundation Fellowship from The Bermuda Biological Station, Inc., a Lerner Fund Grant from the American Museum of Natural History, a grant-in-aid from Sigma Xi, and a Graduate Summer Fellowship from S.U.N.Y. at Stony Brook. This is contribution number 299 from the Program in Ecology and Evolution of the State University of New York at Stony Brook and contribution number 775 from the Bermuda Biological Station for Research, Inc.

SUMMARY

Ascidia nigra is free of epibionts, although many other ascidians are susceptible to epizooic recruitment. Larvae of *Pennaria tiarella* and *Ecteinascidia turbinata,* two epibenthic species found in the same habitats in Bermuda as *A. nigra,* were used in laboratory experiments which demonstrated that the surface of *A. nigra's* test is toxic to settling larvae. There was no evidence that *P. tiarella* or *E. turbinata* is repelled by the toxic surface. The toxicity of *A. nigra* is probably due to the high vanadium content of the surface deposit and to the release of free sulfuric acid from capsules in the test. Both the vanadium-rich surface deposit and the acid-filled capsules are believed to be formed by degenerating vanadocytes in the test. Vanadocytes may also be involved in defense against competitors, parasites, and predators of ascidians.

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COMPARISON OF THE GRADIENT OF SETAL DEVELOPMENT OF UROPODS AND OF SCAPHOGNATHITES IN *ASTACUS LEPTODACTYLUS*[1]

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The intermolt stages of decapod crustaceans are usually defined according to the criteria of Drach (1939; 1944). Many authors subsequently adapted this method to their own material for treating diverse physiological problems. Drach and Tchernigovtzeff (1967) then generalized this technique for application to all crustaceans. The original method allowed rigorous classification, but required injury to the animal due to the extraction of appendages used for microscopic observation. In order to choose from the tremendous number of crayfish available in populations, it was necessary to find criteria for stages within the cycle that would allow a quick and simple identification of molt stages without damage.

The observation of more easily accessible appendages was suggested by several authors: the pleopods of the macruran, *Homarus americanus,* (Aiken, 1973) or of some Stomatopoda (Reaka, 1975); the antenna scales of a natantian, *Palaemon paucidens,* (Kamiguchi, 1968); the uropods of three natantians (Scheer, 1960); or appendages of an anomouran, *Petrolisthes cinctipes* (Kurup, 1964); or of the macrurans, *Homarus americanus,* (Donahue, 1954; Rao, Fingerman and Fingerman, 1973); *Orconectes sanborni* (Stevenson, Guckert and Cohen, 1968) and *Parastacoides tasmanicus* (Mills and Lake, 1975).

The need to keep the animals uninjured together with the fact that they possess neither bladed pleopods nor antenna scales, brought us to use the uropodal blades in crayfish to determine molting stages as well as to predict ecdysis. The data obtained from uropods is set out, and the validity of our method has been tested by a comparative study of the scaphognathites.

MATERIALS AND METHODS

Hardness of the shell

The hardness of different parts of the cephalothorax and the appendages can be estimated by palpation following the classical sequence set out by Drach (1939).

Examination of the uropods

After apolysis, two methods can be used to predict the duration of premolt stages. The first, worked out on *Jasus paulensis* (Vranckx, in preparation), measures the retraction of epidermis which has pulled away from the old cuticle.

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[1] This paper is dedicated in homage to Professor Pierre Drach on his jubilee, and the authors also wish to thank him for critical review of this manuscript.

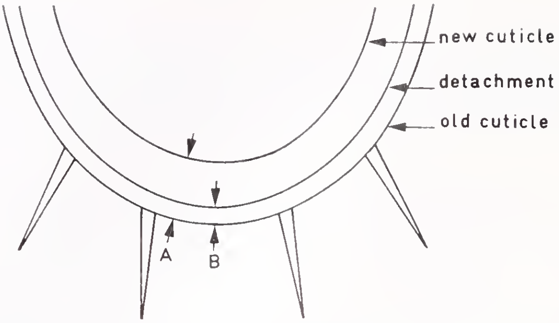

FIGURE 1. Edge of crayfish uropod during the premolt stages showing the measurement of retraction rate index R of the epidermis, which has pulled away from the old cuticle. For a definition of "R", see text.

It is measured with an ocular micrometer, and the retraction rate index R is calculated: R = A/B = distance from external edge of the old cuticle to external edge of the new one/thickness of old cuticle (Fig. 1). To make the measurement easy and to standardize this method, the reading is made on the external edge of the uropod. Care must be taken that detachment of the epidermis, if any, is really related to the premolt state and not to any adjacent injury.

The second method follows the setal evolution in the uropod blades and can be studied with a stereoscopic microscope (Wild × 50). The moistened uropod is set on a rough glass and viewed as a transparency. This determination in *Astacus leptodactylus* is more difficult than in other macrurans because of the thickness and opacity of the uropod blade and its double-bordered setal range (primary and secondary). The internal part of the exopodite of the uropod is examined below the dieresis line between the distal and proximal parts. This thin part of the uropod does not possess the double set of setae.

Comparative study of scaphognathites

Pieces of scaphognathite on both sides of the muscular insertion are picked off and mounted in water with the forepart uppermost. Similarly, the endites of the second maxilla are observed with a light microscope.

RESULTS

Hardness of the shell

The post-molt cuticle that was very soft in stage A gradually becomes harder and harder until the stage C2. In addition, more information can be obtained by feeling the abdomen above the pleurite attachments: it remains flexible crosswise until the end of stage C1.

The shell is hard from C2 through C3 to stage D2, but a certain flexibility can sometimes be noticed from later stage D1 in the branchiostegites within the area underneath the molting line, although this is clearly distinct from stage D3. Then, from stage D3 to ecdysis, the exuvial lines of the shell soften.

It should be noted that after molting the subjects are often brightly colored, tending toward blue throughout stage C3. During this stage the shell is brittle and does not break evenly as in stage C4, when the membraneous layer was being formed. As the animals reach stage C4, the color of the cuticle becomes greenish or brownish, then deepens and turns to a dull color.

Examination of the uropods

Within the uropods, the lacunary circulatory system stays unstructured throughout the cycle, but takes on the appearance of well-structured canals in stage A. These uropods display distinct setal matrices (Fig. 2a). They become homogeneous and opaque up to the end of stage C3. It then becomes impossible to distinguish the perpendicular ribs bordering the uropods in the setae extensions. The position and the structure of these ribs suggest that these can later become matrices or their precursors. Circulating blood cells are not very numerous.

During stage C4, the uropod is more transparent and the thickness of the cuticle is obvious. Furthermore, this stage can be at times subdivided by means of the appearance and development of a light zone that is colorless in the part below the cuticle and whose thickness is similar to the epithelium. Toward the end of stage C4, this zone is bordered by chromatophores. This could indeed represent a transformation of the epithelium. Blood cells are more or less numerous and move about freely throughout the uropods.

In stage DO, the homogeneous aspect disappears, and in compressed uropods the inner fibrillar structures situated in the setae extensions are visible. These formations do not extend beyond one quarter of the distance (D) from the cuticle edge to the first external spine of the dieresis line. They appear like two parallel lines and must correspond to the future matrix (Fig. 2b). To determine stage DO, it is more convenient to seek this very consistent trait, rather than the retraction that is difficult to observe because of the uropod's thickness. Furthermore, especially during stage DO but also sometimes in stage D1′, the hemocytes are less easily transported by the circulation, and they seem to stick to these fibrillar structures more than in other stages.

In stage D1′ the same fibrillar structures are extended and reach half the distance (D) from the shell edge to the first spine. On the other hand, no split can be seen within the thickness of these structures (Fig. 2c).

Due to the splitting of the epidermal tissue, these structures clearly appear in stage D1″ as invaginated setae which extend beyond three quarters of the edge–spine distance (D), and remain clear. On and after this stage where retraction is always obvious, the edge of the new uropod is lightly scalloped (Fig. 2d). Hemocytes are numerous, and they move about. During stage D1‴ the setae reach the bottom of their own invaginations at the distance (D) we previously defined but the setae remain clear, just like the surrounding tissue (Fig. 2e). At times barbules are noticeable in the more transparent parts. Blood cells no longer stick to the matrix or more exactly to the now invaginated seta. The opacity as well as the darkness of the invaginated setae in relation to the surrounding tissue differentiate stage D2 from stage D1‴ (Fig. 2f).

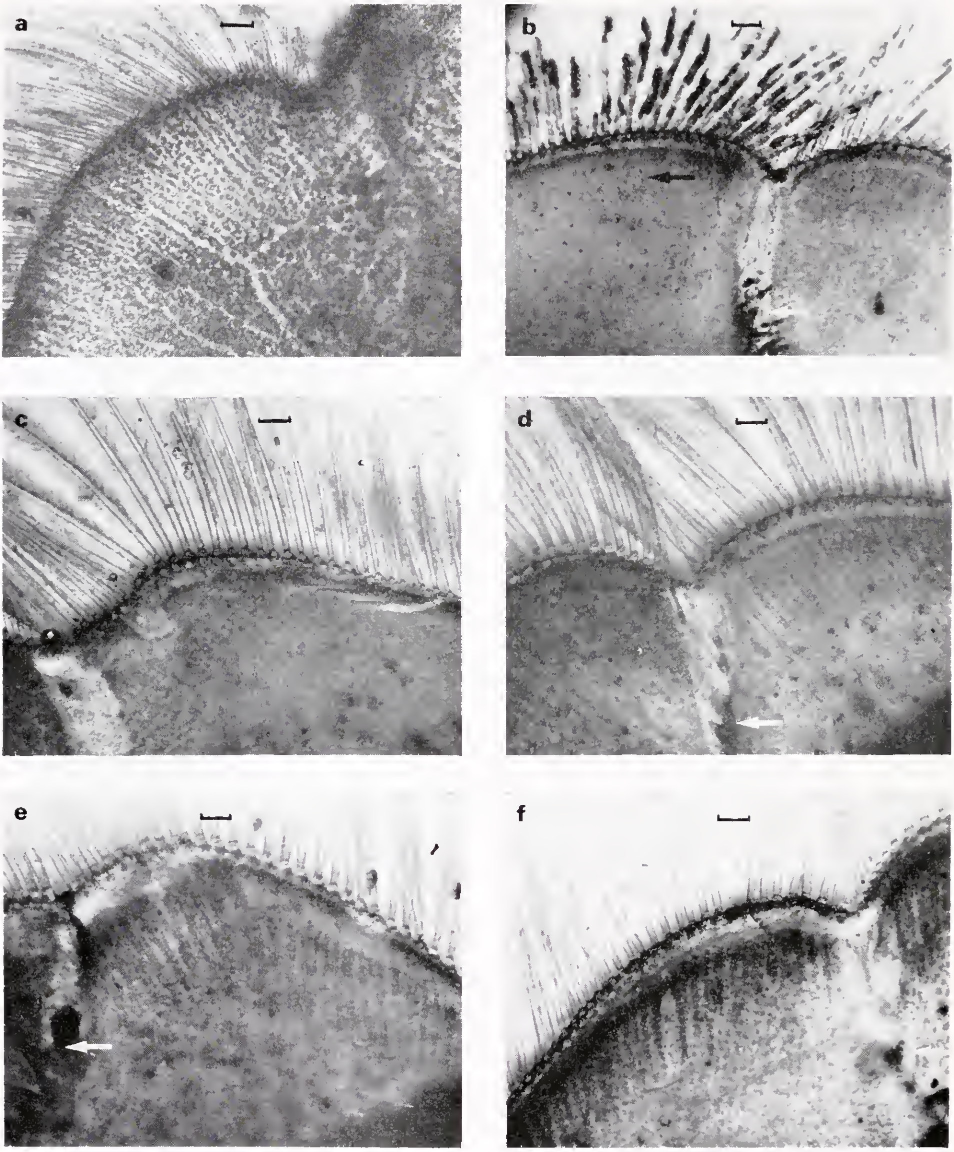

FIGURE 2. Uropod exopodites from *Astacus leptodactylus* examined with a stereoscopic microscope: a, lacunary circulatory system taking on appearence of structured canals in stage A; b, stage DO showing the matrix indicated by the black arrow and the first spine indicated by the white arrow; c, d and e, stages D1′ D1″ and D‴ showing the invaginated setae that progressively extend from the shell edge to the first spine; and f, stage D2 showing opacity and darkness of the invaginated setae. All scale bars equal 0.2 mm.

In stage D3, folds in the underlying integument can be noticed, as Aiken (1973) reported in the lobster. They must not be confused with the scalloped designs formed in the old cuticle. Moreover, there are fewer circulating cells.
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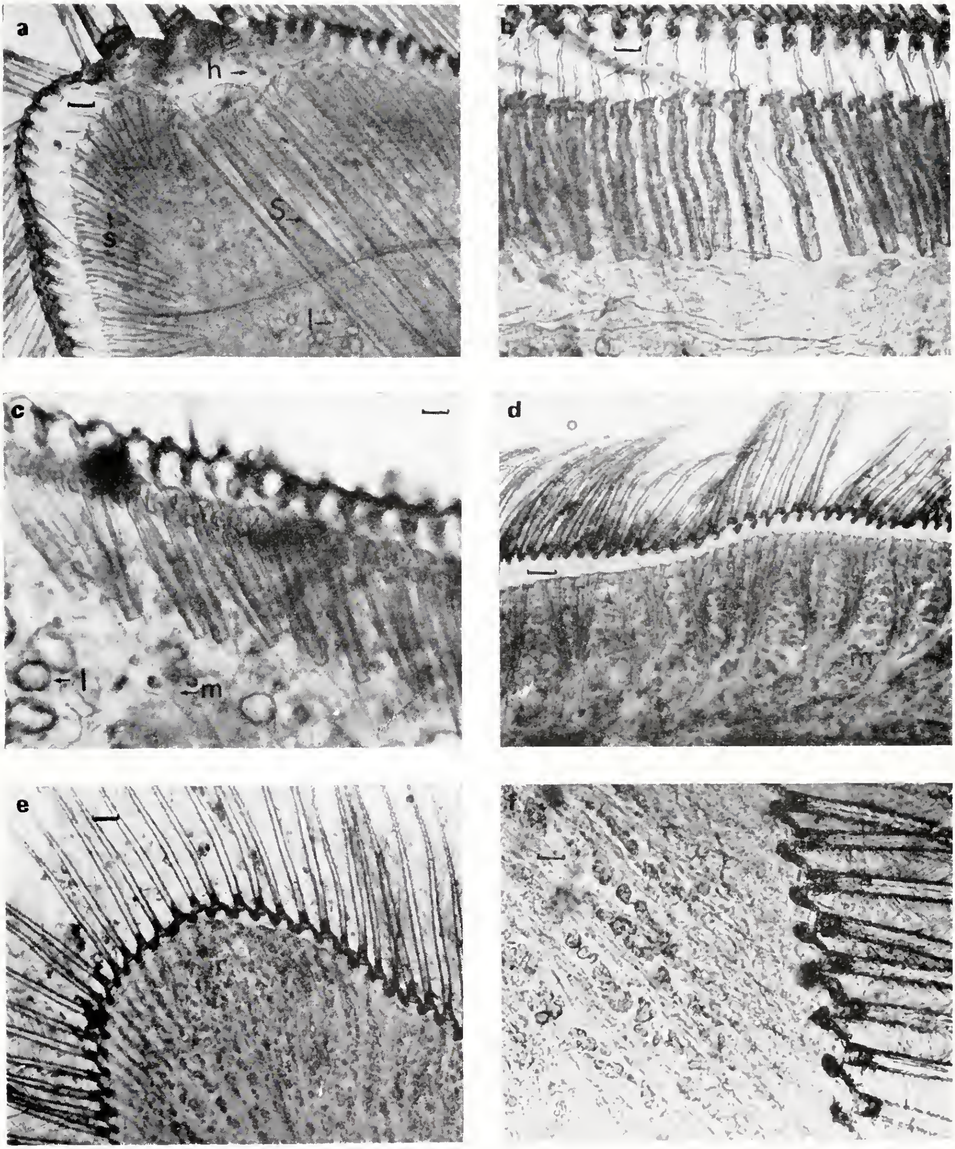

FIGURE 3. Scaphognathites examined with a light microscope (sizes of scale bars in parentheses) : a, epipodite in stage D1''' (150 μ) ; b, setae with single matrix (60 μ) ; c and d, setae with compound or connected matrices (75 μ and 200 μ) ; e and f, stage DO showing matrix swelling (200 μ and 50 μ). Abbreviations are: S, large setae; s, small setae; h, hemocytes; l, "adipocyte" type cells; and m, matrix.

It should be noted that from stage D1 on, when retraction is substantial, the blood cells are often visible in the exuvial space.

Furthermore, the retraction index, showing a progressive retraction from stage DO to stage D4 in *Jasus paulensis* uropods and thus forecasting the date of ecdysis (Vranckx, in preparation), cannot be applied to *Astacus leptodactylus* because of the thickness of the ends of the uropodal blades. In contrast, it can be applied to other Astacidae such as *Procambarus clarkii* (unpublished results).

Study of scaphognathites

From a morphological point of view, the setae bordering the scaphognathite are not altogether similar. Actually, three kinds should be distinguished. First a small number of large setae, situated at the apex of the epipodite, show their matrices (Fig. 3a). In the second kind also, matrices separated from each other at their bases are shown in some small setae (Fig. 3b). Both kinds of setae belong to the type that we call "single matriced". In the third kind the matrices of three to five small setae are clustered together at their bases and are called "compound or connected matrices" (Figs. 3c and 3d).

Even though all setae have synchronous development, examination of the epipodite's two or three biggest ones shows, interestingly enough, that they always seem to be one stage ahead of the others during the D1 phase. The relative discrepancy of staging between biggest and smallest setae is possibly due to the fact that the size of the former renders any phenomenon more obvious (appearance of the fold in stage D1′, barbules). The study of these big setae can be important in clearing up any doubtful cases.

Contrary to the observations made from uropods in which movements of the matrix are seen (*i.e.*, individualization of the matrix from the surrounding tissue or retraction of the Aiken's setal organ) it was impossible to discern any such movement in either of these single or compound matrices within the tissues. From the beginning of postmolt or, in any case, from stage C1 through C2, since this is the first stage studied in a suffcient number of scaphognathites, the matrices or their supports have been set in place. From DO, they swell and thus reduce the intermatrix space (Figs. 3e; 3f).

On the small setae, stage D1″ (Fig. 4b) appears like an intermediate stage of development which happens to be between the stage D1′, the beginning of invagination, and the stage D1‴, in which each invaginated seta attains its greatest depth and has quite obvious barbules (Fig. 3a). In addition, small refractile nodes are seen in D1‴ inside the setae next to the barbule inplantation areas. On the large setae, the barbules can already be distinguished, even though the invagination is not yet finished (*i.e.*, as soon as early D1″). Stage D1‴ can be distinguished from stage D2 not only by means of its more or less strong tissue opacity, as on the uropods, but also by the refringence of the epithelium between the setae (Fig. 3c). Throughout stage D2 to D3 the setae are very clear, but they do not quite reach the bottom of their matrices (Figs. 3c; 3d).

"Adipocyte" type cells, likely the cells of Sewell (1955) are observed inside the scaphognathite. Their size and localization vary during the cycle. These often well developed cells, which reach the edge of the epithelium in stage C1–C2, seem to regress in C3 and C4, and remain thus until the stage DO. They then increase in number in the central part of the scaphognathite, although they remain small. They grow in size until the end of D1‴ or D2, when they become extremely fragile (Figs. 3a, 3c; 4e). From this latter stage until the ecdysis they have been seen disrupting, and they disappear under microscopic observation.

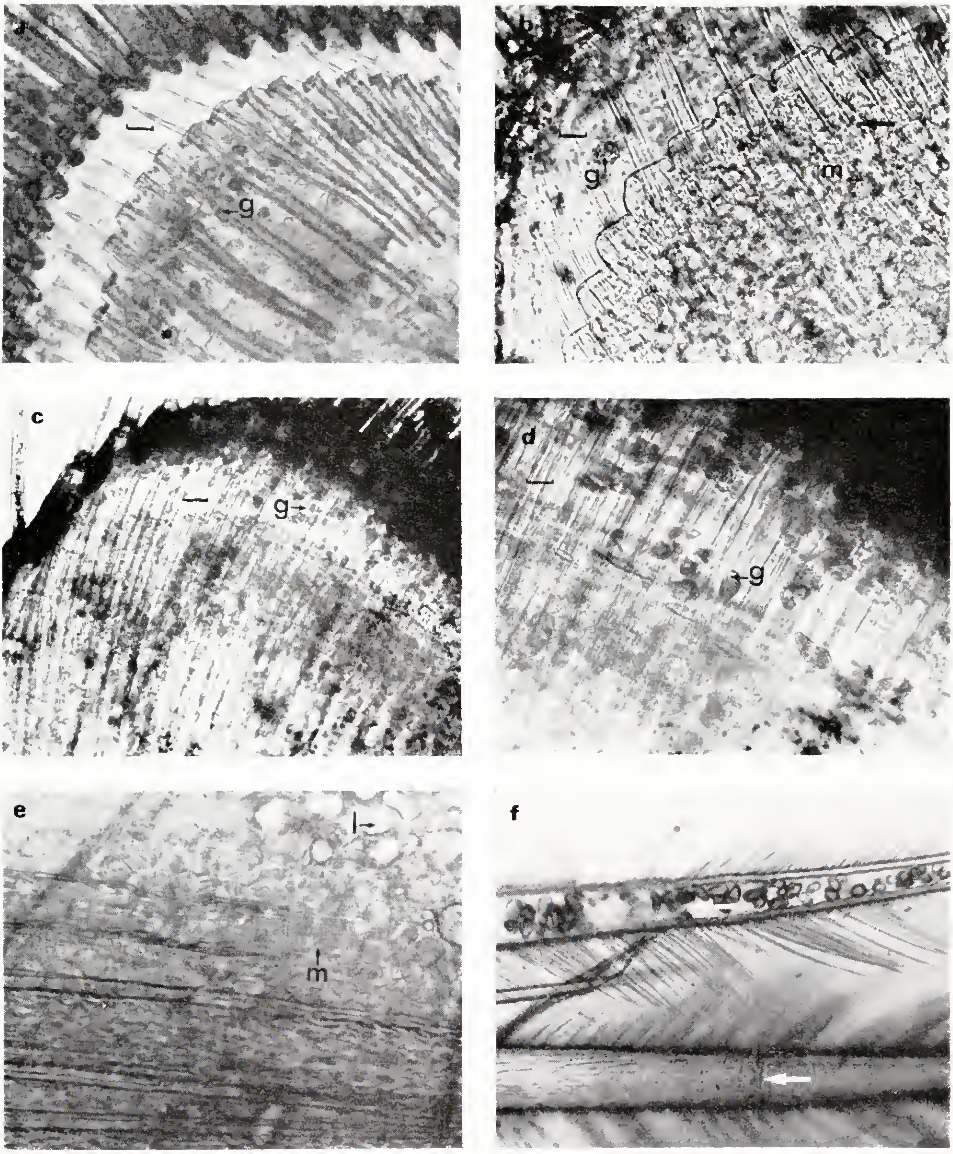

FIGURE 4. Scaphognathites observed with an optical microscope (sizes of scale bars in parentheses): a, scaphognathite in stage D2 (g, represent the granulocytes) (75 μ); b, scaphognathite in stage D1′ (the arrow shows the invagination and m, represents the matrix) (30 μ); c and d, endites from second maxilla, with granulocytes in the exuvial space (100 μ and 30 μ); e, large setae of epipodite and its matrix (shows the "adipocyte" type cells and m represents the matrix); and f, setae with the bottom of invagination fold indicated by the arrow and cellular components unretracted.

The origin of the differences in the "adipocyte" type cell localization is perhaps due to the variations of physiology of each animal studied and to the stages of development of the matrices.

The number of circulating hemocytes varies during the molt cycle. There is an increase of the blood cell density, especially that of granulocytes, in C2–C3, then in D1‴–D2 (Fig. 4a). Moreover, granulocyte accumulations appear between the former cuticle and the new one (Fig. 4b); this phenomenon is especially clear at the end of stage D1, when the maxillary endites are examined (Figs. 4c and 4d).

Durations of the premolt stages

The length of the premolt stages was not precisely evaluated. It can nevertheless be estimated from 4 to 8 weeks, starting from middle DO when proecdysis has already begun. This time bracket is quite long because some animals that are labelled D1′ occasionally molt in the aquarium prior to those labelled D1″ observed at the same time. Although these latter results appear illogical, they may be due to factors in the animals' past lives before we obtained them (injury, environmental factors).

DISCUSSION

The duration of the premolt stages is greater than that indicated by Stevenson *et al.* (1968) for *Orconectes sanborni,* but under different experimental conditions: their animals were much smaller and the water temperature much higher. Mills and Lake (1975) estimated the premolt period at 14 weeks from stage DO, using southern hemiphere, autumn animals. The fact that the molting cycle unfolds with slight differences according to the season may explain their findings. Indeed, in *Astacus leptodactylus* the spring and summer crayfish shift imperceptibly from stage C4 to premolt, and in certain subjects that molt twice during the summer, the stage C4 is reduced to the bare minimum so that C3 tends to go almost directly into stage DO. On the other hand, some autumn animals seem to stop their molt cycle at the beginning of stage DO and not in stage C4, as Aiken (1973) clearly observed in *Homarus americanus,* and Vranckx (unpublished results) also observed in *Jasus paulensis.* For the postmolt animals our findings agree with those of Stevenson (1968).

The division of the intermolt cycle into important substages (Drach, 1939) is mainly determined by morphological characteristics. Drach defined stage D as the premolt period and subdivided it into four substages (D1 to D4).

After further research, another stage, DO, was introduced into the stage D (Charniaux Legrand, 1952), and described by Drach and Tchernigovtzeff (1967) as "the retraction of epidermis and setal matrices." These authors claim this phenomenon is the first morphologically obvious feature showing that the animals have entered the premolt stage. Further studies about the molt physiology have shown obvious variations in results for stage DO, while relatively homogenous data were obtained for the other stages. Growth studies (Aiken, 1973, on *Homarus;* and Vranckx, unpublished results, on *Jasus* and *Astacus*) have shown different kinds of growth curves as the animals enter the premolt phase. These growth curves differ according to the investigated animals and the season in which they enter the premolt. The shape variations of these curves are especially related to kinetics of the DO phase. At this stage, too, the growth rate of the regenerating limb bud varies in *Gecarcinus lateralis* (Tchernigovtzeff, 1972). The great variability of epidermal DNA content in stage DO compared to other premolt stages in *Orconectes sanborni* (Stevenson, 1972) is another fact that could support the idea of a heterogenous stage DO. The heterogeneity of stage DO thus described seems well established, for Drach and Adelung's specifications do not really coincide for this stage (Spindler, Adelung and Tchernigovtzeff, 1974).

This difficulty has brought different authors to subdivide stage DO into two or three substages ("early DO", "middle DO" and "later DO") to explain their results. Once this stage is subdivided, it seems that gastroliths are never found at the beginning of DO in *Astacus leptodactylus* (Vranckx and Durliat, unpublished results); furthermore, Tchernigovtzeff (1965) showed that the mitotic rise was seen in middle or later DO in *Carcinus maenas.*

From all these data, it seems that different physiological phases can be included in a single morphological stage, DO. A first period is characterized by the retraction of the epidermis (beginning of DO, according to several authors) which does not indicate the beginning of premolt, but only the end of integumentary quiescence. So, Skinner (1962) noticed minimal respiratory activity ($\underset{\sim}{Q}O_2$) in crustaceans during stage DO. This raises the problem of whether anecdysis, if any, is found in stage C4 or rather in early DO. Anecdysis in stage DO would explain the precocious detachment of the nail in *Orchestia* (Charniaux-Legrand, 1952) as well as the blocking of certain *Carcinus maenas* by *Sacculina* parasites in C4–DO or DO (Andrieux and Berreur, unpublished results). This may explain why Keller and Adelung's (1970) histological figures of DO in *Orconectes limosus* resemble those of C4. Thus, at the beginning of stage DO, the morphological state (apolysis) is not identified with a physiological state, since detachment is not necessarily followed by ecdysis (Jenkin and Hinton, 1966).

On the other hand, a second phase in DO is characterized by a mitotic rise in the epidermis when the animals truly re-enter the premolt phase. Furthermore, several authors have investigated the action of the ecdysteroids on the onset of the molting activity and our interpretation could explain their data on a new basis. So, Willig and Keller (1973) have evidenced by bioassay that, although in DO characterized by apolysis, 75% of the crayfish do not possess detectable molting hormone, they do not detect ecdysteroids in 50% of animals in stage D1! The effect of ecdysteroids on the molt has been extensively discussed by Rao *et al.* (1973) and even when apolysis is triggered off in some animals by remaining ecdysteroids, for instance the small growth observed following basal growth of the regenerating limb bud in *Sesarma* (Passano and Jyssum, 1963), it is strange that the subject becomes blocked only at the beginning of DO and does not develop further on, with the exception of Stomatopoda (Reaka, 1975). But Gilgan and Farquharson (1977) evidenced that, at a physiological level, it is not only the amount of ecdysteroids that allow the premolt process to proceed further on, but also the tissue sensitivity to the ecdysterone, which depends on the investigated stage and is significantly higher in stage D1′ than in stage C4 or DO. This stage sensitivity is perhaps related to the action of both molting and molt-inhibiting hormones. On the other hand, the fact that in autumn the molt process of our animals does not proceed further than stage DO can not be explained by a reduced sensitivity to ecdysterone at reduced temperature (Gilgan and Burns, 1977), because our animals were kept in a tank at constant temperature. Ecological factors must then intervene in order to re-establish the premolt (Kracht, 1976). In any case, for the physiologist, it may be wiser to consider the morphological stage of DO as a terminal stage of C4. Once activation is obtained, the molting process seems irreversible after the end of DO or the beginning of D1.

When premolt begins, the hemocytes accumulate in the hypodermis and stick to the matrices. They must carry substances needed for the elaboration of the new cuticle and may be even the "activators" for epidermal proliferation, as demonstrated by Bohn (1977) in the cockroach, *Leucophaea maderae*. These cells within the hypodermis should not be confused with the precursors of lipid cells described by Sewell (1955).

Moreover, the passage of blood cells into the exuvial space as observed in insects by Barra (1969), Devauchelle (1971) and Zacharuk (1972) and perhaps noted in the lobster by Hepper (1965), probably happens during apolysis. The hemocytes might contribute to the enzymatic activity of the exuvial liquid.

The setogenesis observed *in vivo* in the scaphognathite of *Astacus leptodactylus* then occurs as Stevenson *et al.* (1968) described, in a "cylindrical zone with lower cell density", whereas in the uropods it is merely observable from middle DO on as a span of cytoplasm that reaches from the top of the future seta "into a cluster of nuclei" in the integument, which correlates with Thomas's observations (1970) in *Austropotamobius pallipes*. Hence, setae organize themselves within an epithelial cylinder (the matrix) according to the pattern described by Tchernigovtzeff (1976), which, incidently, explains Reaka's "schizocoelous slits" (1975). There are not two types of setogenesis as Mills and Lake (1975) claim, but two kinds of quiescent matrices, depending on the species investigated. The matrix is observed either as a "setal organ" at the basis of a seta (contracted form of the matrix when it does not retract out of the lumen of the seta during the intermolt period, *i.e.,* big setae of *Nephrops'* scaphognathite, unpublished result) or as an epithelial cylinder inside the tissue (elongated form of the matrix which is due to its retraction out of the seta, *i.e.,* big setae of *Astacus* scaphognathite). Kurup (1964) and Aiken's (1973) setal organ therefore would not be a special organ, but would simply be due to the preception of nuclei and cell bodies of the matrix which leave the epithelium level and move inwards into the hypodermal tissue during the growth of the seta (Fig. 4e).

During ecdysis, setae evaginate due to the internal pressure of the animal, whereas during metecdysis, the matrices are more or less promptly retracted according to the animal, in a spring-back effect. Thus, the localization of the post-exuvial cuticular secretion of cones, which should not be confused with the trace of the invagination fold, is explained (Fig. 4f). It is true that little discrepancies are obvious, in staging *in vivo,* according to the observation of the different appendages or part of the same one (difference between large and small setae of the scaphognathite). Yet, a conclusion would be premature, since the actual deposit of the different cuticle layers is only revealed by ultrastructural studies. So the epicuticle is already present on the setae in D1′, although it is only visible in D1‴ with an optical microscope (Tchernigovtzeff, personal communication). Unfortunately, Kummel, Claassen and Keller (1970) and Chassard-

Bouchaud and Hubert (1973) do not indicate the exact chronology of the different layers of cuticle.

It is necessary to be very circumspect relative to comparative tables which suggest that the different integumental pre-exuvial layers do not appear at the same stage within the investigated species. The observation of uropods is consequently useful in defining the physiological molt stages, since it establishes the stage DO in *Astacus leptodactylus* after the premolt activation. Moreover, it is especially important for blood studies not to disturb the animal, since a single withdrawal of a small amount of hemolymph induces modification of both the number and type of hemocytes.

Summary

1. Characterization of the different stages of the molt cycle of *Astacus leptodactylus* has been established using morphological criteria of the setae observed through the uropod. This method is paraticularly useful for the scientist who wants to get replicable, physiological data without disturbing the animals.

2. Stages determined from uropod studies agree with those obtained from comparative studies of scaphognathites.

3. According to the seasons, a more or less quiet intermolt phase is observed. The animals either directly enter the premolt phase or are blocked in stage C4 or early DO. Therefore, the morphological stage DO seems to be heterogenous, and apolysis is not necessary to the onset of premolt.

4. A single mode of setal formation seems evidenced. Moreover, setae with compound or connected matrices are seen in the scaphognathite.

5. Number and mobility of blood cells in the uropods are related to the investigated stage. During the premolt stages hemocytes, especially granulocytes, are observed within the exuvial space.

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